

BIOCHEMICAL PROPERTIES OF MICROTUBULAR PROTEIN

IN BRAIN SUBCELLULAR FRACTIONS

by

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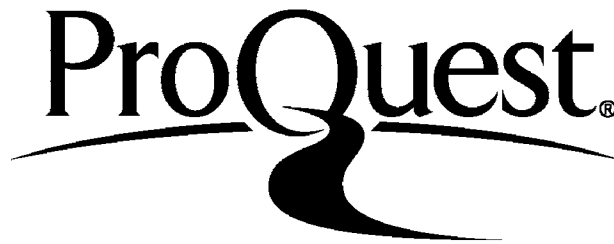
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ABBREVIATIONS

The style of the Biochemical Journal has been adopted in writing this thesis and the following abbreviations are used accordingly:

ATP	Adenosine 5'-triphosphate
CB	Colchicine-binding
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetra-acetic acid
EGTA	Ethanedioxy-bis (ethylamine)-tetra-acetate
GTP	Guanosine 5'-triphosphate
MES	2-(N-morpholino) ethanesulphonic acid
Mit	Mitochondria
MT	Microtubule
My	Myelin
PAGE	Polyacrylamide gel electrophoresis
RSA	Relative specific activity
SA	Specific activity
SDS	Sodium dodecyl sulphate
SPM	Synaptic plasma membrane
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
Vb	Vinblastine



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ABSTRACTBIOCHEMICAL PROPERTIES OF MICROTUBULAR PROTEIN IN BRAIN SUBCELLULAR FRACTIONS.

Chick brain was early shown to be a rich source of microtubular protein, but had not been previously employed to investigate its subcellular distribution.

During the course of this work, subcellular fractions of 1-3 day old chick brain were prepared using different procedures based on floatation-sedimentation centrifugation techniques. The subcellular distribution of microtubular protein was determined by assaying the colchicine-binding (CB) activity in the various fractions obtained.

It was shown that both the soluble and synaptic plasma membrane-containing fractions were enriched in CB activity. A membrane fraction enriched in myelin was also found to bind colchicine, but the CB in this case was atypical since it was not stabilized by vinblastine.

The distribution of vinblastine-binding activity was also investigated. The myelin fraction showed the highest binding activity for this alkaloid.

Furthermore, the polymerization-competence of tubulin present in soluble extracts of synaptosomes, prepared from chick brain according to two different methods, was investigated. Preliminary attempts failed to demonstrate the polymerization of tubulin in such synaptosol preparations incubated under typical microtubule assembly conditions, although SDS-PAGE analysis showed the presence of both  $\alpha$ - and  $\beta$ -like protein bands.

However, incorporation of glycerol and DMSO, as microtubule stabilizing agents, during extraction of synaptosomes carried out at room temperature was found to yield a sedimentable form of CB-protein

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which apparently represented stabilized microtubular structures and accounted for ca. 10% of the total tubulin (CB-protein) content of synaptosomes. Such structures were readily solubilized in the cold and could be reassembled into filamentous elements under standard microtubule assembly conditions. The non-sedimentable form of tubulin (ca. 40% of total synaptosomal tubulin) prepared under these conditions was not polymerization-competent and probably represents the free tubulin subunit pool of synaptosomes. The remainder (ca. 50%) of CB-protein present in synaptosomes was firmly associated with membrane structures.

Filaments reassembled from synaptosome extracts appeared to be enriched in an anomalous slower form of  $\alpha$ -tubulin as judged by SDS-PAGE analysis. In addition, these filaments were enriched in a faster-migrating protein component which migrated slightly ahead of actin.

Possible reasons for the failure to reassemble typical microtubules from synaptosome extracts are discussed.

## Chapter I    Introduction

### 1.1        General features of microtubules

1.1.1      Occurrence and structure of microtubules

1.1.2      Characterization of the microtubular protein

### 1.2        Distribution of microtubules in neurones

### 1.3        Assembly of microtubules

1.3.1      In vivo assembly

1.3.2      In vitro assembly

### 1.4        Alkaloid-binding properties of tubulin

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### 1.5        Subcellular distribution of tubulin-binding activity

### 1.6        Functions of microtubules

1.6.1      Mitotic movement

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### 1.7        Stabilization of microtubules

## Chapter I    Introduction

### 1.1    General features of microtubules

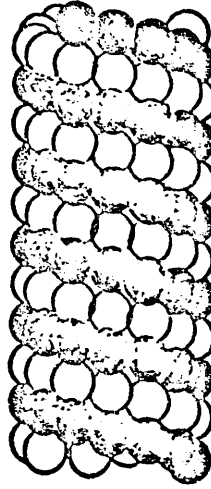
#### 1.1.1    Occurrence and structure of microtubules

Microtubules are a class of organelle whose ubiquity in eukaryotic cells throughout the animal and plant kingdoms is now well recognised. They have been identified by electron microscopy in flagella and cilia, dividing cells, nerve cell dendrites and axons, blood platelets, secretory cells and some specialized organelles of protozoa such as the axostyles of zooflagellates, axopodia of heliozoa, etc.

However, it should be pointed out that even before the advent of the electron microscope, structures now recognised as consisting of interconnected arrays of microtubules, were seen in dividing cells (mitotic spindle apparatus), in nerve cells (together with other classes of filaments making up the 'neurofibrillary apparatus') or in cilia by light microscopy of fixed and stained cells, or in living cells, due to their birefringence (Porter, 1966; Wuerker & Kirkpatrick, 1972; Schmitt, 1968).

As illustrated diagrammatically in fig. 1.1, microtubules seen under the electron microscope, appear as straight, hollow cylindrical structures with an outer diameter of about 25 nm and of variable length, from less than 0.5  $\mu\text{m}$  (in centrioles) to several micrometers in flagella of certain spermatozoa or compound cilia. In cross-section, the wall of the microtubule is about 4-5 nm in thickness, while longitudinally it consists of protofilaments composed of globular units of about 4-5 nm in diameter. A total number of 13 protofilaments was first reported by Ledbetter and

a)  
Microtubule  
surface  
lattice



●  $\alpha$ -subunit  
○  $\beta$ -subunit

b)  
Crosssection  
through a  
microtubule

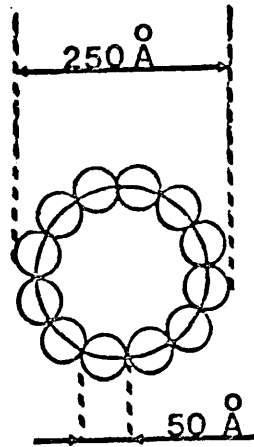


Fig. 1.1

Diagrammatic illustration of the microtubule cylindrical structure. For details see text, section 1.1.1.

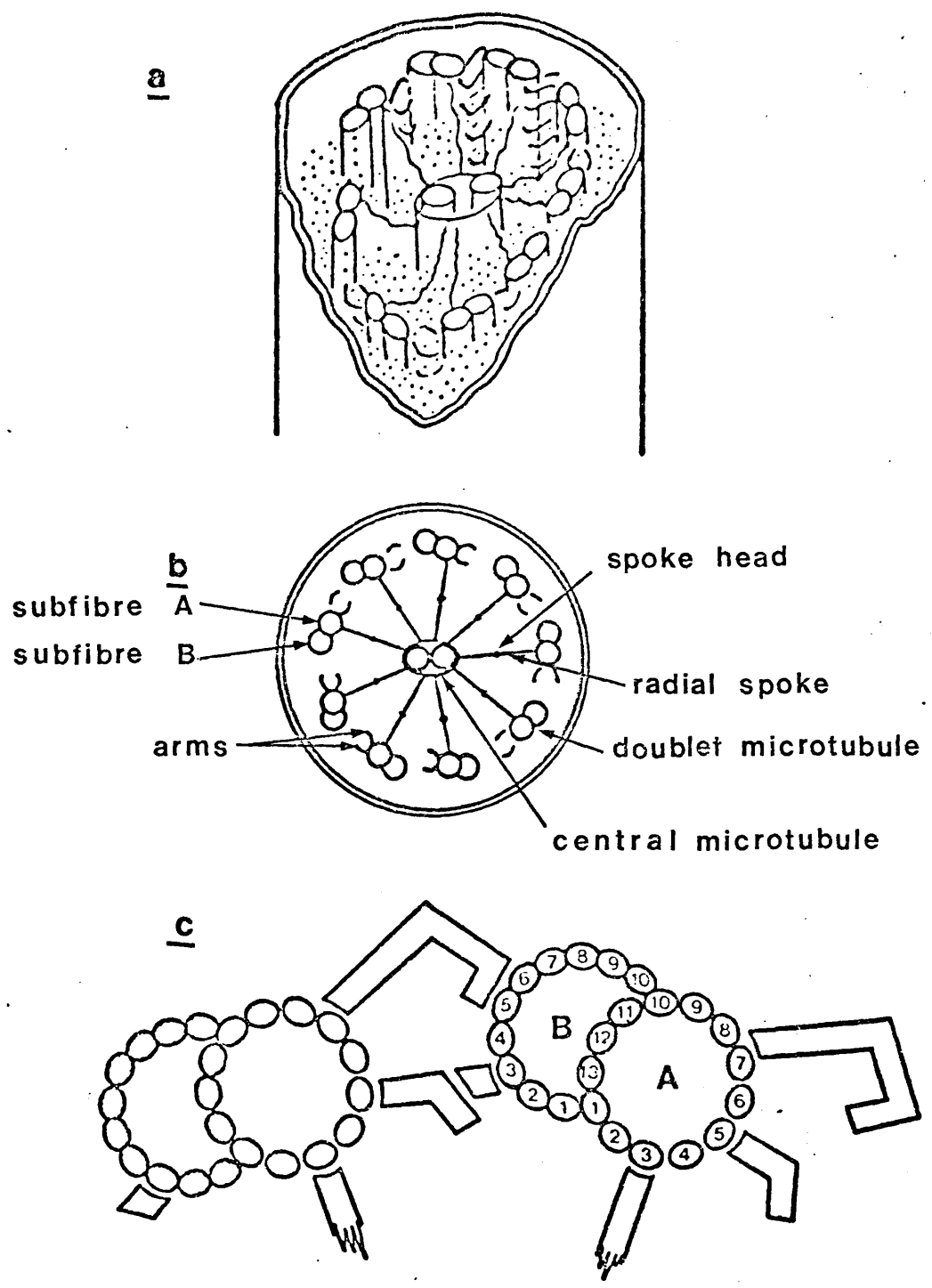
Porter (1964) and has recently been confirmed by Mizuriah & Futaesaku (1971, 1974) and Tilney et al. (1973). However, the existence of microtubules with either 12 or 15 protofilaments has also been reported by Burton et al. (1975).

Stable microtubules are the main fibrous elements in the motile cilia and flagella of unicellular as well as of metazoan organisms, where they are characteristically present in a pattern referred to as the "9 + 2" which is diagrammatically illustrated and described in fig. 1.2. Sophisticated studies combining electron microscopy and biochemical analysis led to the view that in motile organelles, flagellar movements involving bending and beating are based on a sliding mechanism occurring between adjacent pairs of the outer doublet microtubules, driven by cyclical interactions between the ATPase-containing 'dynein' side-arms of the A-tubule subfiber with the B subfiber of the neighboring doublet (see Satir, 1968, 1974; Gibbons, 1975; Summers, 1974; Summers & Gibbons, 1973; Hiramoto, 1974; Gibbons & Gibbons, 1974).

In sensory receptor cells (e.g., retina rod cells), a simplified version of this pattern is seen where microtubules are arranged in a "9 + 0" pattern (Sjostrand, 1953); this led to the suggestion that sensory receptors are a form of 'modified cilia'.

Large numbers of microtubules have also been shown to be present in the cytoplasm of the cutaneous mechanoreceptor cells, or campaniform sensilia, of the cockroach leg. In this case, the inter-tubular links are very rare as compared to those occurring between the individual microtubules and the cytoplasmic plasma membrane, which represent the main type of functional linkage observed (Moran and Varella, 1971).





**Fig. 1.2** Flagellar structure. a) cutaway diagram of flagellum; b) transverse section viewed from basal surface showing the "9 + 2" pattern of microtubules (see text, section 1.1.1); c) arrangement of subunits in outer doublet.

With the introduction of glutaraldehyde, as a fixing agent in electron microscopy (Sabatini *et al.*, 1963), microtubules were identified as normal cellular 'organelles' in eukaryotic cells at interphase. However, the abundance of microtubules in the mitotic spindle apparatus (as many as 3,000 per spindle; see Rebhun & Sander, 1967), made it possible to detect them by electron microscopy, even without glutaraldehyde fixation, even though spindle microtubules are generally regarded as labile structures.

In general, it would appear that microtubules vary considerably in their stability, depending on their origin, and that even within a single cell, several classes of microtubules can be differentiated on the basis of their stability to high and low temperatures, to pH and to the action of proteases (Behnke & Forer, 1967).

#### 1.1.2 Characterization of the microtubular protein

The clue for characterizing microtubular protein was provided by the success of Gibbons (1963) in isolating and characterizing microtubule-associated 'dynein' ATPase from *Tetrahymena* cilia. The stable microtubules present in cilia and in sea-urchin sperm tail axonemes (outer doublets) were the first sources for the isolation of microtubular subunit protein. This was rapidly followed by the isolation of microtubular protein from other sources (e.g. brains) based on the use of colchicine as a specific probe for microtubule subunits (see section 1.4.1)

Working on the isolated outer doublets of sea-urchin sperm flagella, Mohri *et al.* (1967, 1968) found that the microtubular protein there differed from actin, especially in its interaction with myosin and in the nature of its bound nucleotides; and hence they proposed the name tubulin

for microtubular subunit protein.

The early work on tubulin isolation (Shelanski and Taylor, 1967, 1968; Renaud et al., 1968; Stephens, 1968 a, 1970) showed that, in aqueous solution, it exists as a 6S dimer of MW 110,000 - 120,000. The ultracentrifugation of the protein in 8 M urea and its separation on SDS polyacrylamide gel electrophoresis revealed the presence of a single peak or band representing a monomeric form of 50,000 - 60,000 molecular weight. The monomer was tentatively identified with the 4 nm structural unit (see section 1.1) in the microtubule wall (Mohri & Shimomura, 1973; Shelanski and Taylor, 1967, 1968; Weisenberg et al., 1968; Olmsted et al., 1970).

The first indication that tubulin dimer consisted of two distinct forms of the protein of similar molecular weight came from the work of Renaud et al. (1968), who showed that tubulin from ciliary outer doublets dissociated into two bands when electrophoresed on polyacrylamide gels in the presence of 8 M urea. These were first thought to correspond to tubulins originating respectively from the A- and B-tubules. But, soon, it was found that two species of tubulin were present in A- and B-tubules as well as cytoplasmic microtubules. They were also found to be present in roughly equal amounts and were designated as  $\alpha$ - (the slower moving) and  $\beta$ - (the faster moving) tubulin (Witman et al., 1972; Feit et al., 1971b; Everhart, 1971; Bryan and Wilson, 1971; Olmsted et al., 1971), and having molecular weights of about 54,000 - 58,000 and 46,000 - 54,000, respectively (Feit et al., 1971; Olmsted et al., 1971; Lagnado et al., 1972; Raff and Kaumeyer, 1973; Sakai & Kuriyama, 1974; Ohtsubo et al., 1975).

The two tubulins in fact represented peptides of identical molecular weight<sup>s</sup>, and the difference in mobility of the  $\alpha$  and  $\beta$  polypeptides on

SDS polyacrylamide gels in the presence or absence of urea is apparently due to a difference in the net charge of the protein (Bryan, 1974). However, further investigations showed the two tubulins to be different in many aspects; one example is the partial amino acid sequence near the N-terminal (Luduena et al., 1973). This heterogeneity might contribute to differences in stability found amongst microtubules within a given cell (Behnke & Forer, 1967; Linck, 1973), and to immunological differences (Fulton et al., 1971; Yanagisawas et al., 1973) amongst tubulin derived from a single cell type. More recently, Lu and Elzinga (1975) reported the separation of three forms of tubulins ( $\alpha_1$ ,  $\alpha_2$ ,  $\beta$ ) by column chromatography of brain microtubule protein on hydroxyapatite under denaturing conditions (see Chapter VII).

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 stabilized 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).

## 1.2 Distribution of microtubules in neurones

Microtubules have been described as constant components of nerve cells, and thus neuronal organelles, where they are implicated in the movement of substances within the cytoplasm, a process called cytoplasmic flow (Schmitt, 1963). This cytoplasmic movement occurs also in the

dendrites (Globus et al., 1968) and cell body (Pomerat et al., 1967), where indeed most of the protein synthesis in the nerve cell takes place. The early observation showed that microtubules end short of the synaptic contact in an axon with a single 'bouton terminal', and avoid the whole synaptic complex in the case of multiple contacts or 'bouton en passant' (Gray and Guillery, 1966; Wuerker and Kirkpatrick, 1972). The recent evidence of a close association between microtubules and synaptic structure, and their preservation in synaptosomes (see Gray, 1975; Bird, 1976; Hajos, 1976) will be described below.

Microtubules are very abundant in thin axons and the distal ramified parts of the dendrites, whereas the fibrous elements in large myelinated axons consist mainly of neurofilaments (100 Å in diameter) distinct in their chemical structure from microtubules (Huneus and Davidson, 1970; Gilbert, 1975). Furthermore, the cytoplasm of the dendrites, which have the highest surface to volume ratio, is filled with a striking number of long, slender unbranched microtubules (Gonatas & Robbins, 1965). They are characterized by a straight equidistant arrangement which gives the interior of the dendrite a lattice-like appearance as revealed by electron micrograph cross sections (Wuerker and Kirkpatrick, 1972).

So far, there is little evidence for a direct structural link among the microtubules or between them and other cellular elements. However, recent studies, using lanthanum staining techniques, revealed the presence of amorphous 'filamentous' extensions on the surface of microtubules in various invertebrate nerve cords (Burton and Fernandez, 1973). Such extensions would presumably participate in intertubular links. It was also suggested that neurotubules and neurofilaments are involved in a three dimensional network (Metuzals, 1963).

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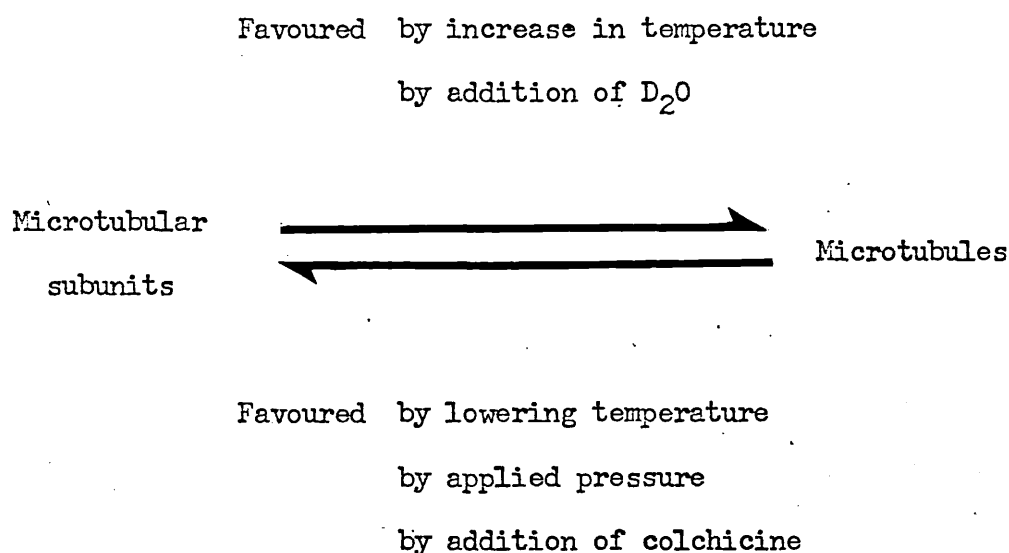
The early studies of microtubules in nerve endings showed that they do not form part of the synaptic complex and that they end short of the synaptic contact (Gray and Guillery, 1966; Peters et al., 1970). Based on the overlapping observed between microtubules and vesicles, some authors suggested that vesicles were formed by budding from microtubules (Pellergino de Iraldi and De Robertis, 1968). Very recent studies have shown a clearer and more realistic close association of microtubules with synaptic vesicles, presynaptic dense projections, and smooth reticulum-like membranes in the axon terminals (Gray, 1975, 1976; Bird, 1976). Further investigations also showed that microtubules in mature and immature central nervous system synapses, exist in close relationship with, or are apparently attached to certain membrane specializations underlying the postsynaptic membrane, including postsynaptic 'thickenings' that are sometimes referred to as postsynaptic densities (PSDs) (Westrum and Gray, 1977). Such observations are in keeping with immunocytochemical and biochemical studies showing that tubulin is a major protein of synaptic junctional complexes (e.g., Banker et al., 1974; Matus et al., 1975; Walters and Matus, 1975; Kadota et al., 1976). Based on the above described association between microtubules and postsynaptic 'thickenings', Westrum and Gray suggested, that microtubule may play an important role in the initiation and formation of the synapse, and its maintenance later when it is mature.

### 1.3 Assembly of microtubules

#### 1.3.1 In vivo assembly

The lability of cytoplasmic microtubules present in nerve processes is well established and can be correlated with their existence in a state

of dynamic equilibrium with their subunits (tubulins). In an elegant series of experiments, using a sensitive polarising microscope, Inoue (1952, 1953 and 1964) studied the effects of various factors on the stability of mitotic spindle microtubular bundles in various types of living cells, by birefringence changes. He found that temperatures below  $10^{\circ}\text{C}$  induced a reversible loss of spindle fiber birefringence. The return to normal temperature restored the birefringence in the course of a few minutes, while increasing the temperature to  $37^{\circ}\text{C}$  brought about a maximum increase in birefringence. Furthermore, this reversible disintegration of the mitotic spindle was also observed when the preparation was treated with colchicine (see diagram below).



Later, when the thermodynamic aspects of this process were investigated (Inoue and Sato, 1967), it was found that assembly was an endothermic process associated with a large increase in entropy. Such data are in keeping with earlier experiments concerning the thermodynamic aspects of monomer-polymer transformation during the polymerization of actin (Asakura *et al.* 1960) and of tobacco mosaic virus coat protein (Lauffer,

1958).

Since it was suggested that most of the birefringence was accounted for by the ordered array of microtubules in the spindle (Luyks, 1970), it was generally assumed that alteration in birefringence reflects changes in microtubule assembly. In particular, it has been proposed that the disruption of microtubules by colchicine, and other antimitotic alkaloids, is created by shifting the equilibrium towards the monomeric state following the binding of the drug to free microtubular subunits (Borisy and Taylor, 1967).

The findings that protein synthesis inhibitors (e.g., actinomycin and chloramphenicol) do not prevent the reappearance of birefringence following the removal of the spindle disrupting agent (colcemid) by several washings (Inoue and Sato, 1967) suggested that the reformation of the mitotic spindle fibers was due to the presence of a pool of readily available subunits and not to de novo synthesis of monomers. Based on these findings, a dynamic equilibrium theory was proposed by Inoué. He suggested that microtubule monomers and polymers existed in an equilibrium state which is naturally altered during the formation of the mitotic spindle. This same concept was also found to apply for the reversible assembly-dissassembly of microtubules studied in the axoneme of Actinosphaerium (Tilney and Porter, 1967).

The precise factors involved in initiating microtubules in vivo and in controlling the extent and direction of their growth, are all at the moment rather hazy. However, a number of observations, now available, makes it possible to support the concept of microtubule organizing centres or MTOCs as referred to by Pickett-Heaps (1969). Such centres or nucleating sites, as they have also been called, are not necessarily represented



by rigidly defined organelles or electron microscopically observed structures. Several workers have postulated the existence of MTOCs on theoretical and observational grounds (Tilney, 1968; Porter, 1966; Inoue and Sato, 1967), and in one case they have even been equated with small amorphous electron-dense areas associated with the microtubule ends. However, it should be emphasized that there is no clear and convincing indication yet of the identity of the MTOC material. Cautiously, MTOC could be considered as small, inconspicuous dynamic structures (Roberts, 1974).

### 1.3.2 In vitro assembly

The early work on the isolation and characterization of tubulin produced enough information to enable several investigators to search for the appropriate conditions under which microtubules could be polymerized in vitro. The first attempts were performed on purified brain tubulin in the presence of GTP since it was shown to be bound to tubulin and to stabilize its colchicine binding properties (Borisy et al., 1972). Under such conditions, non-tubulin filamentous aggregates, occasionally beaded, were observed and were found to be temperature and GTP dependent. At about the same time, Weisenberg (1972) reported the first successful microtubule assembly in vitro from crude rat brain extracts in the presence of GTP, indicating that the maintenance of low concentrations of free  $Ca^{++}$  was mandatory to achieve the assembly in vitro of patent microtubules. Indeed, excess  $Ca^{++}$  was later shown to promote disassembly of formed microtubules in vitro. Following this initial report, studies have been directed toward characterizing the polymerization reaction. These involved:-

a) Establishing ionic and buffer requirements

It was found that polymerization occurs maximally at moderate ionic strength, 0.15 M for  $\text{Na}^+$ , 0.1 mM - 1 mM for  $\text{Mg}^{++}$  and  $\text{Ca}^{++}$ , near neutral pH, 6.0 to 7.5, at physiological temperature, 25 - 37°C and in the presence of GTP (Olmsted and Borisy, 1973; Lee et al., 1974; Olmsted and Borisy, 1975). The minimal protein concentration (i.e., total protein) at which polymerization in brain extracts could occur was about 4 mg/ml.

b) Developing procedures for purifying assembly-competent microtubule protein

Shelanski et al. (1973) reported that the presence of 4 M glycerol or 1 M sucrose in the assembly buffer markedly enhanced the polymerization of tubulin. The microtubules obtained under these conditions were found to be much more stable to low temperature and colchicine treatment. The removal of glycerol or sucrose resensitized the microtubules to cold and colchicine effects. The yield in pure tubulin was over 90% when two cycles of assembly in glycerol at 37°C and disassembly in the absence of glycerol were performed.

c) Examining the role of accessory proteins in the polymerization process

The presence of a number of minor protein components copurifying with tubulin had been shown to be independent of the presence or absence of glycerol during assembly. These components comprised high molecular weight proteins (MW ca. 300,000 - 350,000), which represented up to 15% of the total protein, medium molecular weight proteins (MW ca. 110,000 - 170,000) and an actin-like protein (MW ca. 45,000 - 48,000). It has been

found that these copurifying proteins are essentially required for microtubule assembly (Murphy and Borisy, 1975; Weingarten et al., 1975). It is possible that these accessory factors interact directly with tubulin subunits to form a short segment of the microtubule lattice which then serves as a nucleating centre.

d) Presenting evidence that microtubule polymerization proceeds by a nucleated assembly mechanism

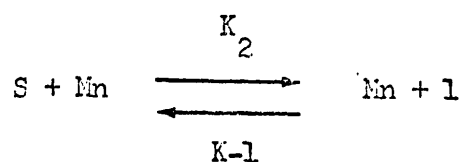
It was early shown (Borisy and Olmsted, 1972), that tubulin in high-speed supernatant from porcine brain would not polymerize under the conditions described by Weisenberg (1972), but would readily do so when present in low-speed extracts. The examination of the low-speed extracts by sedimentation technique and electron microscopy revealed the presence of 30 S particles which were identified as 290 Å-diameter disc-like structures. At a temperature favoring tubule formation, the amount of these 'rings' would decrease, and increase when existing microtubules are depolymerized by cold or other means. This led to the suggestion that the discs might be incorporated into the forming microtubules and may act as nucleating centres to initiate microtubules polymerization.

Furthermore, experiments, based on sedimentation velocity analysis and aimed at determining the protein at equilibrium and the reactions that determine the equilibrium concentration of each species, revealed the presence of two components: a rapidly sedimenting species (> 300 S) corresponding to microtubule polymer, and a slowly sedimenting species corresponding to the 6S tubulin heterodimer (Johnson & Borisy, 1975; Johnson, 1975). Following the sedimentation of the polymer, the only detectable species remaining in solution was the 6S tubulin. Thus, it

was tentatively identified as the monomer unit with respect to the polymerization reaction.

To show that the 6 S species was in equilibrium with microtubules, some samples were first polymerized at high protein concentration, then diluted to allow depolymerization, and others were initially polymerized at the lower concentration. In both cases, the determination of protein distribution in the final equilibrium state gave the same amounts of monomer and polymer.

Further experiments to characterize the polymerization reaction involved the determination of the distribution of protein into monomer and polymer fractions as a function of the total protein concentration. The findings revealed the existence of a critical protein concentration below which no polymer was formed, and that the initiation of polymer formation begins with an abrupt transition step, at total protein concentration above this critical value. The amount of polymer formed was found to be proportional to the total protein concentration, whereas the concentration of monomer in equilibrium with polymer was found to be independent of the total protein concentration when this one was above the critical value. Experiments in which the microtubules were sheared revealed that the equilibrium concentration of 6 S tubulin above the critical concentration was independent of the mass, average length and number concentration of polymer. These results were accounted for by a type of reaction referred to as a condensation polymerization mechanism according to which elongation occurs by the consecutive addition of monomer units to the end of the microtubule (see also illustrated scheme in fig. 1.3). The equilibrium would be written as:-



where S is the subunit,  $M_n$  and  $M_n + 1$  are microtubules consisting of  $n$  or  $n + 1$  subunits, respectively, and  $K_2$ ,  $K-1$  are the rate constants for association and dissociation. In this case, the equilibrium constant  $K_e$  would be equal to  $\frac{1}{S}$ .

In addition, these results implied the need of a distinct nucleating step to initiate microtubules formation.

e) Characterizing protein species present under depolymerizing condition or during the early stages of polymerization

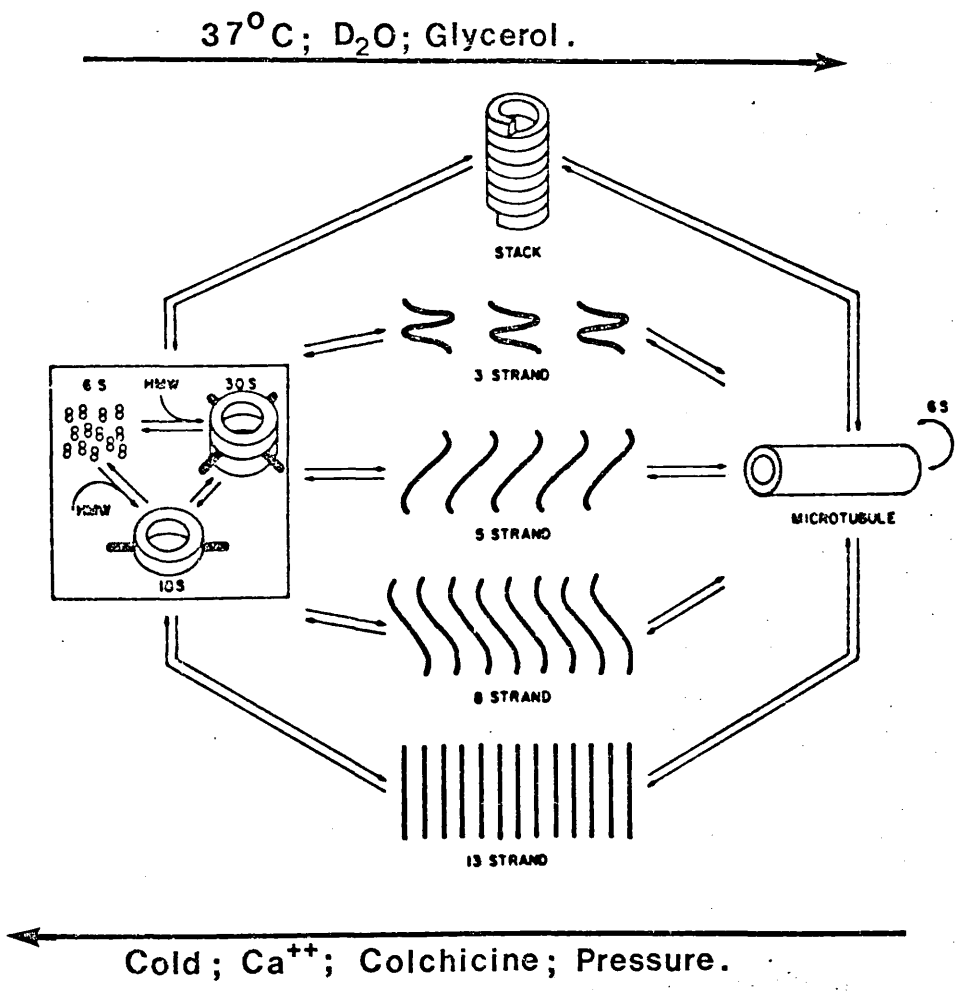
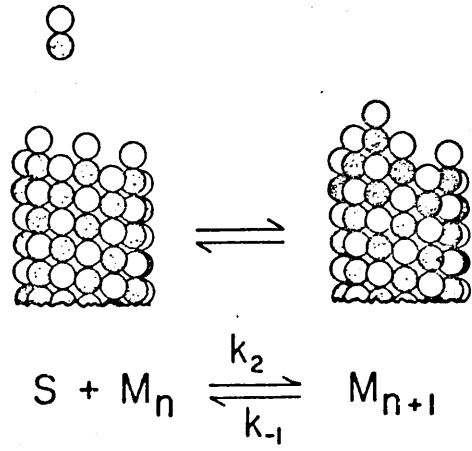
Electron microscopic examination of the tubulin forms, during the early stages of polymerization, revealed the presence of ribbons or sheets of up to 12 protofilaments (Erickson, 1974). It was suggested that the elongation of these sheets, during polymerization, resulted from the addition of subunits and protofilaments. Later, it was found that depolymerization of microtubules at  $0^\circ\text{C}$  gave rise to both 6 S dimers and curved or ring-shaped structures (Erickson) which were separated chromatographically, and their properties studied in relation to the polymerization process. The ring forms were found to reassemble rapidly into microtubules after uncoiling into protofilaments, which in turn formed the tubule wall through lateral association. The 6 S would not assemble into microtubules on its own, but was found to readily incorporate into tubules in addition of some ring 'seeds' (see fig. 1.4). Similar observations were also reported by Kirschner et al. (1974, 1975).

It is noteworthy that this assembly model as described by Erickson

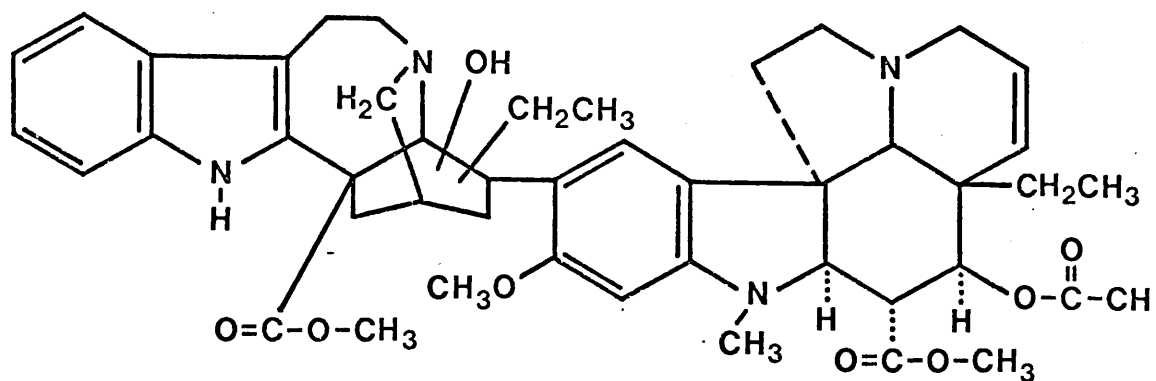
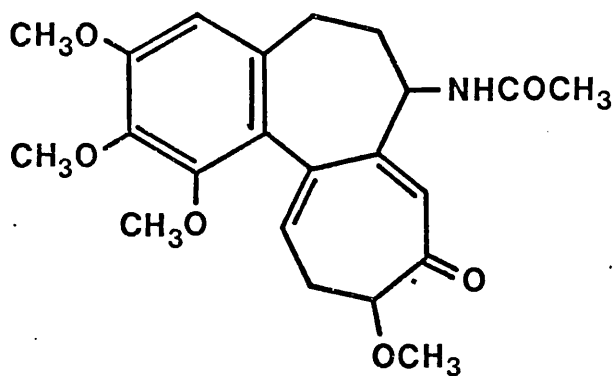


Fig. 1.3 Microtubule assembly scheme (Johnson; see text, section 1.3.2 d). Diagrammatic representation of microtubule polymerization by association of 6S tubulin dimer subunits at the end of the microtubule. S, subunit;  $M_n$ ,  $M_{n+1}$ , microtubule consisting of n or n + 1 subunits respectively;  $k_2$  and  $k_{-1}$ , rate constants for association and dissociation, respectively; (modified from Borisy et al. 1976).

Fig. 1.4 Summary scheme of the microtubule assembly pathways (Erickson; Kirschner et al.; see text, section 1.3.2 e). The box on the left contains the solution species thought to be at equilibrium at low temperature. The 18S and 30S oligomers are represented as single and double rings, respectively. The helical stack and various helical strands represent possible assembly intermediates thought to derive either by uncoiling of the ring oligomers or by association of the 6S dimer and non-tubulin accessory proteins (HMM). After the nucleus is formed, elongation of the microtubule proceeds by addition of 6S subunits preferentially at one end (modified from Borisy et al. 1976).





**A** Vinblastine**B** Colchicine**Fig. 1.5**

Chemical structures of vinblastine (A) and colchicine (B).

See also text, sections 1.4.1 and 1.4.2.

and Kirschner, is not characterized by distinct phases of nucleation and elongation, as in the case of the model proposed by Borisy and co-worker. Furthermore, the suggestion by Johnson that elongation of microtubules occurs by the addition of 6 S dimer subunit to the growing end of the microtubule, is also different from Kirschner and Erickson proposals, where the polymerizing unit is primarily the uncoiled oligomer (cf. fig. 1.3 and 1.4).

#### 1.4 Alkaloid-binding properties of tubulin

##### 1.4.1 Colchicine

The selective binding of the mitotic spindle inhibitor colchicine (see fig. 1.5 A for structure) to the microtubule protein is now well established (Borisy and Taylor, 1967) and is being used as a means to purify and assay tubulin from various sources (Weisenberg et al., 1968). The general idea is that each tubulin dimer (6 S; 120,000 MW) has one colchicine binding site (Shelanski and Taylor, 1968). This concept was further supported by other investigators (e.g., Wilson, 1970), and additional findings that the monomer itself does not usually bind colchicine were reported together with data showing that the dimer loses rapidly its binding ability by undergoing conformational changes in its structure (Ventilla et al., 1972).

The colchicine binding process is a reversible reaction with an optimum temperature of 30°C and optimum pH of 6.8-7.0. The dimer binding capacity is generally lost in the intact tubule except possibly at the free 'ends' of microtubules (Margolis et al., 1978).

Furthermore, it is now generally accepted that the disrupting effect

of colchicine on cytoplasmic microtubules is due to its binding to the free soluble dimer, thus shifting the thermodynamic equilibrium in favour of microtubule disassembly by removing the available pool of active subunits.

Recent studies also show that the reduced thiol groups are essential for the binding reaction and that high concentrations of glycerol or sucrose greatly increase the half-life of tubulin colchicine-binding activity independently of the bound nucleotides (Solomon et al., 1973).

Other antimitotic drugs (e.g., the Vinca alkaloids, vinblastine and vincristine) were found to stabilize colchicine-binding activity, most probably through binding at a separate site (Wilson, 1970).

On the other hand, a significant colchicine-binding activity was found to be also associated with the membrane fractions of brain extracts (Lagnado and Lyons, 1971; Lagnado et al., 1971), where it was suggested to interact with a stabilized and membrane-bound form of tubulin. Similar high colchicine-binding activity was also found in chromatin and membrane fractions of liver cell homogenates (Stadler and Franke, 1972). Further reports by the same workers (Stadler and Franke, 1973) revealed a distinction between tubulin and membrane-bound colchicine-binding fraction in liver, as luminocolchicine (a photoisomer of colchicine) binds to the membrane-bound protein but not to tubulin; however, they showed that in the case of brain extracts, very little luminocolchicine was bound to the membrane fraction, further supporting the view that in this tissue, membrane bound activity was probably due to tubulin-like material.

#### 1.4.2    Vinblastine

Another alkaloid whose binding properties to tubulin have been much

explored, is vinblastine (see fig. 1.5 B for structure). Although it seems to be less specific in its binding properties than colchicine (Wilson *et al.*, 1970), it is used as a standard means of tubulin preparation as it precipitates tubulin both in vivo (in the form of intracellular paracrystals (Bensch and Malawista, 1969; Behnke and Forer, 1972), and in vitro from tissue homogenate supernatants (Weisenberg and Timasheff, 1970; Marantz and Shelanski, 1970). The relative yield of vinblastine-induced paracrystals was shown to increase in the presence of colchicine (Straus and Sato, 1973), while the precipitation reaction in vitro was found to be controlled by the relative amounts of protein, vinblastine and  $Mg^{++}$  present (Weisenberg and Timasheff, 1970).

No clear picture has yet emerged concerning the chemistry of the vinblastine tubulin-binding reaction. Several investigations have revealed that 1 mole of vinblastine binds two moles (240,000 g) of tubulin (Weisenberg and Timasheff, 1970; Berry and Shelanski, 1972; Owellen *et al.*, 1972). This, together with the suggestion that the release of GTP from the non-exchangeable site is essential for the precipitation of tubulin by the drug (Berry and Shelanski, 1972), has led to the postulation that vinblastine may link two dimers, possibly by their non-exchangeable nucleotide site. The exchangeable nucleotide is not released and half of it is protected from exchange during precipitation.

### 1.5 Subcellular distribution of the tubulin-binding activity

Early work on the subcellular distribution of colchicine-binding activity present in rat brain homogenates showed that a significant proportion of the colchicine binding was located in a fraction enriched

in synaptic plasma membranes (Lagnado et al., 1971). More recent evidence substantiates the view that colchicine binding in subcellular fractions from mammalian brains is mainly associated with isolated synaptic junctional complexes, as well as perikaryal and synaptosomal soluble fractions (Lagnado et al., 1975).

Furthermore, investigation of the distribution of vincristine - binding activity in rat brain homogenates (Tan, 1975) revealed that the binding was mainly associated with the particulate fraction, i.e. nuclear, mitochondrial and microsomal, decreasing in that order. This same work also showed that amongst the lysed crude mitochondrial subfractions, the soluble extract had the highest binding activity for both colchicine and vincristine, whereas the myelin fraction contained a high vincristine-binding activity as compared to a relatively low one for colchicine.

## 1.6    Functions of microtubules

The widespread occurrence of microtubules in cells and organisms suggests that they are involved in many cellular functions. Their characteristic arrangement in cilia, flagella and sperm tails (see section 1.6.2), for instance, signify their involvement in the motility of these active structures. During cell division, the chromosomes movement is very much dependent on the integrity of the microtubules forming the mitotic spindle apparatus. In elongated cells such as nerve cells, microtubules could play a double role. On the one hand, they act as a cytoskeletal support to the cellular processes; on the other hand, they are involved in transporting, from the cell body to the distal parts of the cell, all structural, functional and metabolic material needed for the growth, maintenance and function of the cell. Some aspects of

microtubular functions will now be described in greater detail.

### 1.6.1 Mitotic movement

The molecular mechanism of mitosis in higher eukaryotic cells is largely unknown. Very recently, a new model of mitotic movement based on intrinsic microtubule behaviour was proposed (Margolis et al., 1978). It was based on two events: poleward-sliding of anti-parallel microtubules and an opposite end assembly/disassembly of all microtubules, both occurring constantly throughout mitosis and acting coordinately to produce mitotic movement.

The authors have envisaged a constant assembly of microtubules at the equatorial region of the spindle and at the kinetochores, coupled with a poleward migration of subunits within the microtubules as they flow towards the disassembling ends. In each half spindle, the microtubules have a parallel orientation, whereas inter-polar microtubules, i.e. microtubules originating near or at the pole and non-attached to a kinetochore, from each half spindle overlap in the equatorial region in an anti-parallel fashion. A constant poleward sliding of anti-parallel microtubules past each other works against the kinetochore linkage to produce an isometric tension that is transformed to work by the uncoupling of sister chromatids in anaphase. A system of constant microtubule assembly and disassembly, of anti-parallel microtubule sliding, and of parallel microtubule linkages produces an organelle whose structural integrity is maintained by the opposition of forces in a state of dynamic tension.

### 1.6.2 Motility

The role of microtubules in cilia, flagella and sperm tail movements

was previously referred to during the description of their structure and pattern in such organisms (see section 1.1). It is generally assumed that the tubules of the "9 + 2" complex slide over one another, using energy derived from the hydrolysis of ATP. The process being probably mediated by the ATP-ase arms, or dynein arms that are attached to the A-tubules of outer doublets (Gibbons & Gibbons, 1973; Satir, 1974). Furthermore, the direct involvement of microtubules in motility is supported by the finding that colchicine, through its disruptive activity on cytoplasmic microtubules, led to a loss of oriented movement and extension of pseudopodia in cultured cells (e.g., fibroblasts; see Goldman, 1971).

### 1.6.3 Cytoskeletal

Many microtubule disruptive factors, such as low temperature (Tilney and Porter, 1967), high pressure (Tilney et al., 1966), urea (Shegenaka et al., 1971) or colchicine (Tilney, 1968), had been shown to create a retraction in the axopods of Actinosphaerium. The effects of pressure and low temperature were prevented by D<sub>2</sub>O which was also found to react as a stabilizer of spindle microtubules. Furthermore, the addition of low concentrations of colchicine to growing nerve cells was found to selectively prevent the outgrowth of axonal and dendritic processes (Seeds et al., 1970; Yamada et al., 1970). Such observation confirms clearly the role of microtubules in the formation and maintenance of assymetric cell structures.

### 1.6.4 Axoplasmic transport

Microtubules are a very common and abundant structure in nerve cells, where they are referred to as neurotubules. Their role in such highly specialized cells has been the subject of a vast amount of research.

Also present in nerve cells alongside the neurotubules, is another class of fibrous elements, the neurofilaments, which are apparently composed of a subunit protein quite distinct from tubulin (Anderton et al., 1976).

Within the elongated nerve cell, materials are transported from the cell body to the axonal (and dendritic) extremities. Such transport has been found to occur at two different rates, a specialized fast flow of certain materials along defined cytoplasmic channels (200 - 500 mm/day) and a slow mass flow of materials (1-2 mm/day) (Jeffrey and Austin, 1974).

The involvement of neurotubules in the fast flow mechanism has been suggested by several investigations. Banks et al. (1971) showed that colchicine simultaneously disassembled tubules and restricted the fast flow of noradrenalin storage granules within the axon. Similar observations on the disruption of tubules and axoplasmic flow of protein were reported by England et al. (1973). Schmitt (1968) presented a model in which the vesicle movement along the axons involved a series of cross-bridges with microtubules. Smith et al. (1970) showed a series of pictures in which synaptic vesicles were arranged parallel to, and close to, arrays of neurotubules. Such associations of microtubules with vesicles transported in the axoplasm, were later suggested on the basis of autoradiography studies by Lentz (1972). Furthermore, the involvement of neurofilaments in fast transport was as well suggested (Yamada et al., 1971; Ochs, 1972) and a 'transport filament model' was described in this connection.

Recent evidence indicates that microtubules and neurofilaments are themselves transported in the slow component (Hoffman and Lasek, 1975). Such studies were supported by labelling experiments, which suggest that



the slow component may represent the continuous movement of a network of axonal cytoskeletal element (i.e. microtubules and neurofilaments), judged by SDS polyacrylamide gel analysis of material undergoing transport along the length of the axones as a function of time.

#### 1.6.5     Sensory transduction

The suggestion that microtubules are involved in sensory transduction (Porter, 1966; Schmitt and Samson, 1968) is based on the association between cilia and sensory receptors such as occurs in olfactory cells (Reese, 1965), vertebrate rods and cones (Sjostrand, 1953) and various insect receptors (Gray and Pumphrey, 1958). Later work showed a close functional association of microtubules with the plasmalemma in a sensory organ of the cricket. Moran and Varela (1971) came out with more direct evidence by establishing the failure to evoke action potentials in the mechanoreceptors of cockroach leg (campaniform sensillum) when microtubule-disrupting agents (i.e. colchicine or vinblastine) were applied.

#### 1.6.6     Hormone and transmitter release

Several investigations on the effect of colchicine and Vinca alkaloids on secretion suggest that tubulin may be involved in the release of insulin from  $\beta$  cells of the pancreas (Lacy et al., 1968; Malaisse et al., 1971) of thyroxin and iodine from the thyroid gland (Williams and Wolff, 1970) and of catecholamines from adrenal medulla (Poisner and Bernstein, 1971).

The blockage of synaptic transmission by colchicine was also related to the disruptive effect, on the axoplasmic transport, induced by the drug (Perisic and Cuenod, 1972; Robert and Cuenod, 1969). However, this does not rule out the possibility that nerve terminal membrane may as

well be the site of action of these antimitotic drugs. Katz (1972) has observed a decrease in the mean quantal content of the end-plate potential (epp) of the frog sartorius muscle, under both colchicine and podophyllotoxin, thus suggesting an interaction of these drugs with the plasma membrane. Using similar neuromuscular junction preparations, Turkanis (1973) observed a significant decrease in the miniature end-plate potentials (mepp) when colchicine and vinblastine were used.

### 1.7 Stabilization of microtubules

It is generally thought that various cytoplasmic microtubule-based functions depend on the state of polymerization of the protein, i.e. on the extent to which microtubules are present as tubules or free subunit. To validate this assumption, it would be necessary to assess the proportion of tubulin that is polymerized in tissues according to their functional state. Most biochemical work simply measures the total tubulin content present in soluble extracts prepared in the cold; i.e. tubulin derived from polymers (cold labile), from the free subunit pool (Pipeleers et al., 1977 a; 1977 b) and perhaps also, tubulin which is loosely associated with membranes in situ. Recent observations indicate that a number of stabilizing agents can apparently preserve microtubules during the extraction of tissue, in particular when such extraction is carried out at room temperature.

The most powerful amongst these agents is the solvent dimethylsulfoxide (DMSO). It has been used to stabilize and subsequently isolate microtubules from brain homogenates (Filner and Behnke, 1973). In vitro polymerization of tubulin was also shown to occur more rapidly when DMSO was present in the medium and at an optimum concentration of 10%.

microtubules were stabilized against the depolymerizing action of cooling and high ionic strength (Filner and Behnke, 1973; Dulak and Crist, 1974). Very recently it was reported that tubulin purified from bovine brain, would reassemble into microtubules at concentrations as low as 1 mg/ml in the absence of other associated proteins if 10% DMSO is included in the medium (Himes et al., 1977). Therefore, such an agent is potentially useful to estimate the ratio of polymerized and free tubulin dimers in tissue extracts.

## Chapter II      General Methods

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## Chapter II    General Methods

### 2.1    Introduction

This chapter describes all the general methods used throughout the course of this experimental work. Any alterations introduced to such methods and any other specific method used for a particular purpose are described separately in the appropriate chapter.

#### 2.1.1    Chemicals

All the standard chemicals used in this work were of the highest purity available. All radioactive chemicals were obtained from the Radiochemical Centre, Amersham Bucks, U.K. Tritiated vinblastine was a gift from the same firm. Non-tritiated vincristine and vinblastine were also gifts from Eli Lilly Co., Ltd., Basingstoke, U.K. Unlabelled nucleotides were supplied by Boehringer Corporation Ltd. Other drugs used will be specified in the text.

The purity of labelled drugs was periodically checked by thin layer chromatography as described in section 2.5, and appropriate corrections were made in the determination of specific radioactivities, in the use of tritiated compounds, to account for the loss of label through exchange reactions in aqueous solvents (see Oldham, 1968; Evans et.al. 1970).

#### 2.1.2    Preparation of crude high-speed brain extracts

Brains excised from etherised animals (rats or 1-3 day old chicks) were kept on ice while clearing them from blood vessels and meninges. The tissue was weighed (referred to as wet weight) and homogenized in

9 volumes or 1.5 volume (for 10% & 40% homogenates respectively) of an ice-cold solution of 0.1 M 2-(N morpholino) ethanesulphonic acid (MES) buffer pH 6.5 containing 1 mM ethylene glycolbis-(aminoethyl-ether-tetra-acetic acid (EGTA), 1 mM GTP and 0.5 mM MgCl<sub>2</sub>. This was achieved by using a motor-driven glass homogenizer with Teflon pestle (clearance 0.003-0.005 in). The homogenate was centrifuged for 60 min at 100,000 g (using an MSE SS40) to yield a soluble fraction or high-speed extract from which tubulin was isolated.

## 2.2 Tubulin purification

Tubulin was prepared from brains of various species such as guinea pig, rat, and chicks. The most commonly used species was chick (1-3 day old) since such animals were readily obtainable from the Open University. The whole purification procedure was carried out between 2-4°C unless otherwise specified. The method used was based on that described by Shelanski et.al. (1973), in which tubulin was derived from in vitro reassembled microtubules (see following section); occasionally tubulin was partially purified for identification purposes, by precipitation with Vinca alkaloids (see section 2.2.2).

### 2.2.1 Polymerization method

This was based on the method first described by Shelanski et.al. (1973). Freshly excised rat or 1-3 day old chick brains were freed from blood vessels and homogenized with 1.5 vol. ice-cold assembly buffer (0.1 M MES 1 mM EGTA, 0.5 mM MgCl<sub>2</sub>, 1 mM GTP pH 6.9) in a glass homogenizer fitted with a motor-driven Teflon pestle (200 rpm) by 10 up and down strokes.

The homogenate was centrifuged at 100,000 g for 60 min at 4°C and the resulting supernatant (S<sub>1</sub>) was mixed with an equal volume of assembly buffer containing 8 M glycerol. Following the incubation of the mixture at 37°C for 30 min, to allow the reassembly of the tubulin, the preparation was pelleted at 100,000 g for 60 min at room temperature. The pellet (P<sub>2</sub>), enriched in microtubules, was resuspended in a volume of ice-cold reassembly buffer equivalent to one third of the first supernatant (S<sub>1</sub>) fraction by gentle homogenization (3-4 strokes by hand) in a Teflon-glass homogenizer. After standing on ice for 30 min, to allow the disassembly of microtubules, the suspension was centrifuged at 100,000 g for 60 min at 4°C. This resulted in the sedimentation of a particulate material (P<sub>3</sub>), consisting mainly of non-tubular aggregates as revealed by electron microscopy (L.P Tan unpublished observations). The corresponding supernatant (S<sub>3</sub>) was highly enriched in depolymerized microtubules or tubulin. For further purification of such tubulin, (S<sub>3</sub>) was mixed with an equal volume of assembly buffer containing 8 M glycerol, incubated for 30 min at 37°C and centrifuged under the same conditions as (S<sub>1</sub>), (see centrifugation scheme, fig. 2.1), to yield a pellet (P<sub>4</sub>) consisting mainly of polymerized microtubules. The purity of the preparation was assessed by polyacrylamide gel electrophoresis, as shown in fig. 2.2. and electron microscopy (see fig 2.3).

### 2.2.2 Precipitation with Vinca alkaloids

In the present work, this particular technique was used as a means to detect the presence of tubulin rather than as a purification method for the microtubular protein itself.

It was found that the addition of 10<sup>-3</sup> M vinblastine (Vb) to puri-



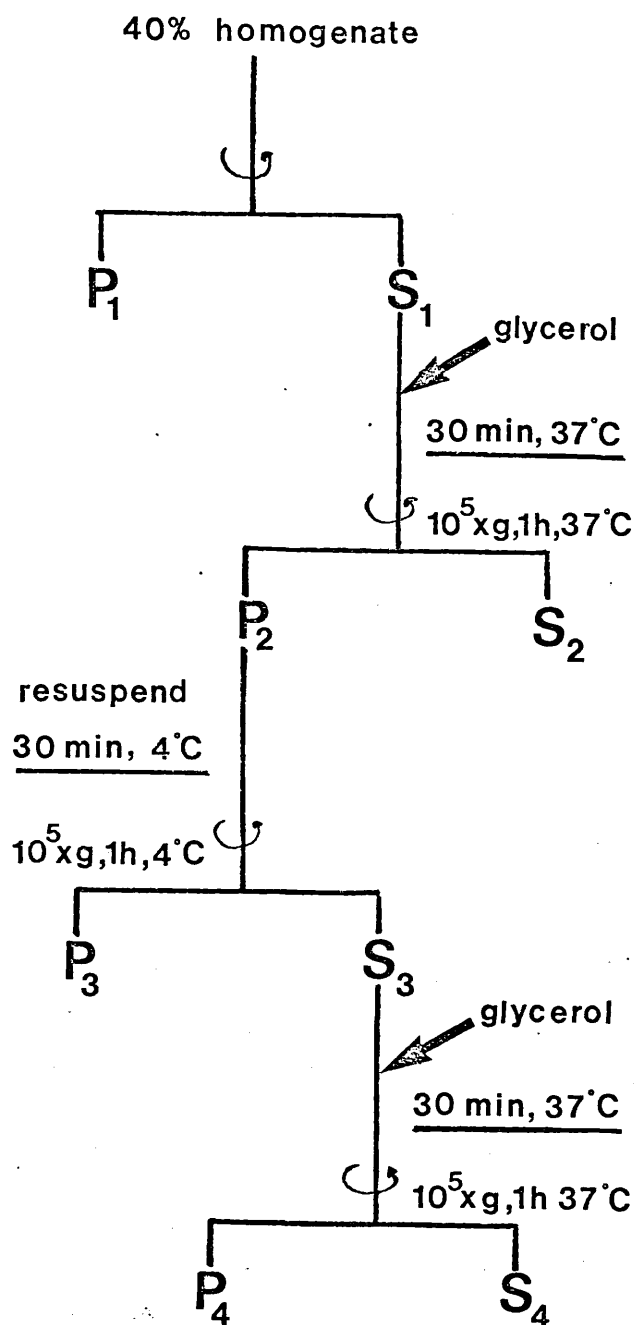


Fig. 2.1 Scheme for purification of microtubular proteins by one cycle of assembly/disassembly, based on the Shelanski method (see text, section 2.2.1). Pellet and supernatant fractions are designated as **P** and **S** respectively.

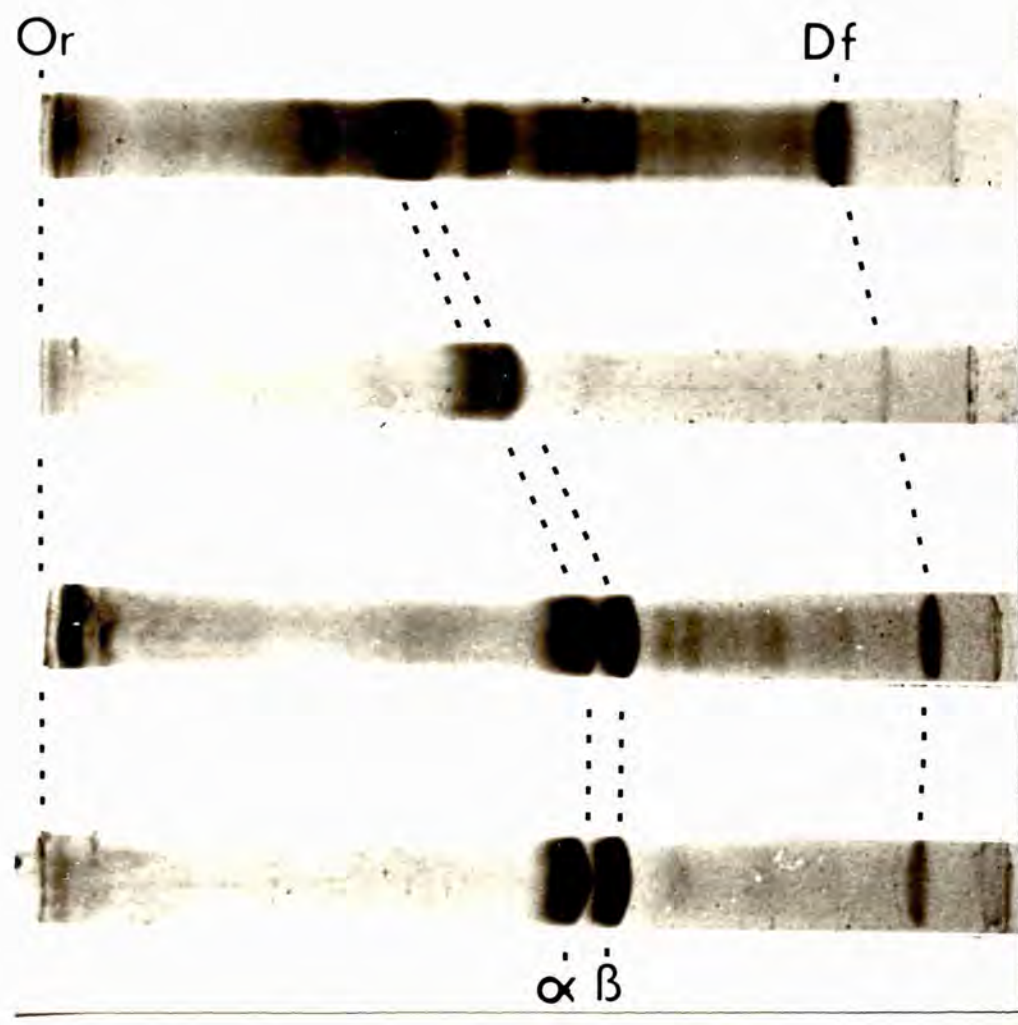


Fig. 2.2 Polyacrylamide gel electrophoresis patterns of samples obtained during the purification of microtubular protein (see text, section 2.2.1) from chick brain, by one cycle of assembly/disassembly in the presence of glycerol. Electrophoresis was carried out in tris-glycine buffer system in the presence of urea (see text, section 2.7).  $S_1$  = initial high-speed supernatant.  $P_2$ ,  $P_3$  and  $P_4$  refer to pellets obtained subsequently as indicated in fig. 2.1 (see also text, section 2.2.1). Note in fraction  $P_4$ , the final microtubular pellet, the minor high molecular weight components (EMW) near the origin (Or), as well as the main tubulin ( $\alpha$  and  $\beta$ ) bands. Df = dye front.



Fig. 2.3 Electron micrograph of a negatively stained sample of  $P_2$  obtained during the polymerization of microtubules in vitro (see fig. 2.1 and text, section 2.2.1). The corresponding polyacrylamide gel is shown in fig. 2.2. Magnification = 7 K.

fied microtubule proteins or to high-speed supernatants from pig brain, resulted in the rapid appearance of a fine precipitate. It also induced the precipitation of about 99% of the colchicine-binding activity present in either preparation (Marantz et.al. 1969). In the present experiments, samples containing about up to 5 mg of protein/ml were incubated for 30 min at 37°C with 500  $\mu$ M (final concentration) of the Vinca alkaloids (vinblastine or vincristine) during which the solution became faintly turbid. After cooling, the incubation mixtures were centrifuged at 100,000 g for 60 min at 4°C. The pellet and supernatant fractions thus obtained, as well as the corresponding fractions from the control sample incubated without the Vinca alkaloid, were assayed for colchicine-binding activity by the charcoal method described in section 2.3.3 (below) and the proteins in each fraction were analysed by SDS-PAGE and compared to the pattern purified by the previous assembly method (see section 2.2.1).

### 2.3 Determination of colchicine-binding activity

The fact that colchicine binds specifically to tubulin provides a suitable means for determining the amount of tubulin present in tissue extracts. When samples containing tubulin are incubated with  $^3\text{H}$ -colchicine, a tubulin-colchicine complex is formed. Various methods are available to separate such complexes from free  $^3\text{H}$ -colchicine; they involve: column chromatography on Sephadex 100, selective absorption of the complex onto Sephadex DEAE A 50, or onto DE 81 filter disc (Whatman), and free colchicine retention by charcoal. Amongst these, only the last two methods were employed in the present work (see sections 2.3.2 and 2.3.3 for description).

The amount of tubulin was indirectly measured by the radioactivity



retained in the sample, on the basis that each tubulin dimer binds up to one mole of colchicine (Weisenberg et.al. 1968). This, of course, assumes that tubulin represents a single species of protein homogenous with respect to its ability to bind colchicine.

### 2.3.1 Incubation conditions

The medium in which the protein (up to 500  $\mu$ g in the final mixture) was assayed for colchicine binding contained 10 mM sodium phosphate buffer with 5 mM  $MgCl_2$  adjusted to pH 6.5. The reaction was started by the addition of  $^3H$ -colchicine to give a final concentration of 5  $\mu$ M in an incubation mixture volume routinely kept at 0.5 ml. The incubation was carried out for 90-120 min at 37°C in the dark to prevent any light induced degradation of colchicine to luminocolchicine ( $\alpha$ ,  $\beta$ ), a derivative which does not show high affinity binding to tubulin (Wilson and Freidkin, 1966). The reaction was stopped by cooling the mixture on ice for 10 min and aliquots of 0.1 ml were used for determination of the protein-bound radioactivity by one of the methods described below. When vinblastine (Vb) was used to stabilize the colchicine binding, a small volume (up to 50  $\mu$ l) of non-labelled Vb was added to the reaction mixture to give a final concentration of 50-100  $\mu$ M.

### 2.3.2 DE 81 filter disc assay

This method was adapted from that described by Wilson (1970) and Lagnado et.al. (1971). Whatman DE 81 filter discs (25 mm in diameter) were numbered and placed on a perspex tray with small hollow cylindrical supports having the same diameter as the disc. The whole tray was placed on ice and the filter discs were moistened with ice-cold washing medium containing 10 mM sodium phosphate buffer and 5 mM  $MgCl_2$  adjusted to pH 6.8.

An aliquot of 0.1 ml from the incubation mixture was pipetted onto two superimposed filter discs and kept on ice for 10 min to allow the absorption of protein-bound radioactivity. The discs were then immersed in a beaker containing ice-cold washing medium (30 ml per disc) which was changed four times, at 5 min intervals, in order to remove unbound  $^3\text{H}$ -colchicine. The discs were then blotted briefly on tissue paper and transferred to vials containing 5 ml of Bray's scintillation fluid for counting (see section 2.8.1 below). All assays were performed in triplicate.

### 2.3.3 Charcoal absorption assay

This method is based on the removal of free  $^3\text{H}$ -colchicine by absorption to charcoal as it was first described by Sherline et.al. (1974).

Following the stoppage of the colchicine-binding reaction (see section 2.3.1), aliquots of 0.1 ml of the sample reaction mixture were added to an equal volume of 0.2% (w/v) ice-cold albumine solution using small plastic tubes immersed in ice. A volume of 1 ml of ice-cold activated charcoal suspension (5-10 mg/ml distilled water swirled vigorously prior to each pipetting) was added to each tube and the total mixture thoroughly shaken. After it was allowed to settle on ice for 10 min, the suspension was centrifuged at 2,200 g for 10 min in a BECKMAN B microfuge kept in the cold. Aliquots of 100-500  $\mu\text{l}$  of the supernatant were then added to 5-10 ml of Bray's scintillation fluid for counting (see section 2.8). The control in such assay contained the same concentration of  $^3\text{H}$ -colchicine, but without added microtubular protein.

### 2.3.4 Comparison of assay methods

The two methods of assay described above were compared in order to

assess their reliability. In this connection, samples from rat brain high-speed supernatant containing up to 500  $\mu\text{g}$  of protein were incubated in the presence of  $^3\text{H}$ -colchicine for 90 min at  $37^\circ\text{C}$  (see section 2.3.1). Triplicates of 0.1 ml were assayed by the DE 81 filter disc assay and activated charcoal methods, respectively. The results obtained showed that the variations between the triplicates in the DE 81 methods ranged between 10 and 25% of the mean value, whereas those of the charcoal method never exceeded 5-10%.

Such high variations, in the case of the first method, could easily result, either from the washing process where small fragments from the filter discs are lost due to partial disintegration of the paper material, or during the blotting process where some of the protein-colchicine complexes could have been absorbed by the blotting tissue. Furthermore, the ease and rapidity of the charcoal absorption procedure makes the processing of multiple aliquots of each sample more practical for routine assays.

Finally, the controls resulting from the charcoal method proved to be very low, in terms of counts per minute, as compared to those obtained by using the filter discs, which were shown to retain high counts of  $^3\text{H}$ -colchicine. This retention could be solved by including non-tritiated colchicine in the buffer used to moisten the discs, but at the same time could create a situation where the "cold" colchicine could exchange with the tritiated one in the protein-colchicine complex.

The possibility of retaining protein complexes by the charcoal was reduced by adding the 0.2% albumin solution to the assaying mixture. Thus, the charcoal method proved to be far more suitable than the DE 81 filter disc assay, but unfortunately it was introduced at the last stage of the present work.

#### 2.4 Determination of vinblastine-binding activity

In this case, the same components used in the colchicine reaction mixture were included except that the binding reaction was started by the addition of  $^3\text{H}$ -vinblastine to give a final concentration of  $5\ \mu\text{M}$ .

The incubation time was reduced to 15-20 min at  $37^\circ\text{C}$ , and the reaction was terminated by immersing the tubes in ice. An aliquot of 0.1 ml from each sample was transferred onto a DEAE impregnated filter disc (Whatman DE 81) which had been previously mounted on the Millipore filtration apparatus and moistened with ice-cold 10 mM phosphate buffer, containing 5 mM  $\text{MgCl}_2$  and adjusted to pH 6.8. The filter disc was washed three times, each with 10 ml of the same ice-cold buffer, under mild suction. Each disc was counted in 5 ml of Bray's scintillant. Triplicate determinations were carried out on each sample, and the blank consisted of samples incubated on ice for the same period of time.

#### 2.5 Thin layer chromatography

The purity of  $^3\text{H}$ -colchicine and  $^3\text{H}$ -vinblastine was checked routinely by thin layer chromatography analysis to detect any impurities or degradation compounds that might have resulted from storage.

##### 2.5.1 $^3\text{H}$ -colchicine

Labelled colchicine was chromatographed in the presence of pure colchicine ( $50\ \mu\text{g}$ ; Sigma) on 0.3 mm thick silica gel G (Kieselgel; Merk) plates (20 x 20 cm). The plates were developed with methanol as described by Wilson and Friedkin (1966) and viewed, once dry, under ultra-violet (UV) light to locate the colchicine and any other impurities. Usually, one spot corresponding to the colchicine was detected with an  $R_f$  value of 0.56 under



the above system. Direct counting of the gel scrapes (1 cm<sup>2</sup> per scintillation fluid-containing vial) along the colchicine spot, showed that more than 95% of the radioactivity recovered was associated with the authentic colchicine spot revealed under UV.

### 2.5.2    <sup>3</sup>H-vinblastine

Chromatography was carried out on Eastman Kodak chromatogram sheets (60 61), 5  $\mu$ l of <sup>3</sup>H-vinblastine and 20  $\mu$ g unlabelled vinblastine were applied to the chromatogram which was developed in a chloroform-methanol (1:1) solvent system. The chromatogram sheets were then dried and sprayed with Dragendroff's reagent prepared according to the method described by Meunier and Machebœuf (1951). Most of the Vinca alkaloids (present in the range of 20-50  $\mu$ g) react with this reagent to give an easily detectable orange colour.

In the present case, a strip of the chromatogram containing the pure unlabelled Vb spot (Rf = 0.89 under the present system) was cut into 1 cm sections and counted directly in Bray's scintillation fluid. Counts were corrected for any quenching due to the presence of supporting material, which decreased the counts by 10-20%. The purity of the tritiated Vb was determined according to the percentage of recovered counts found to comigrate with the authentic Vb. This usually ranged between 90% and 95%.

## 2.6    Protein estimation

### 2.6.1    Determination of protein contents

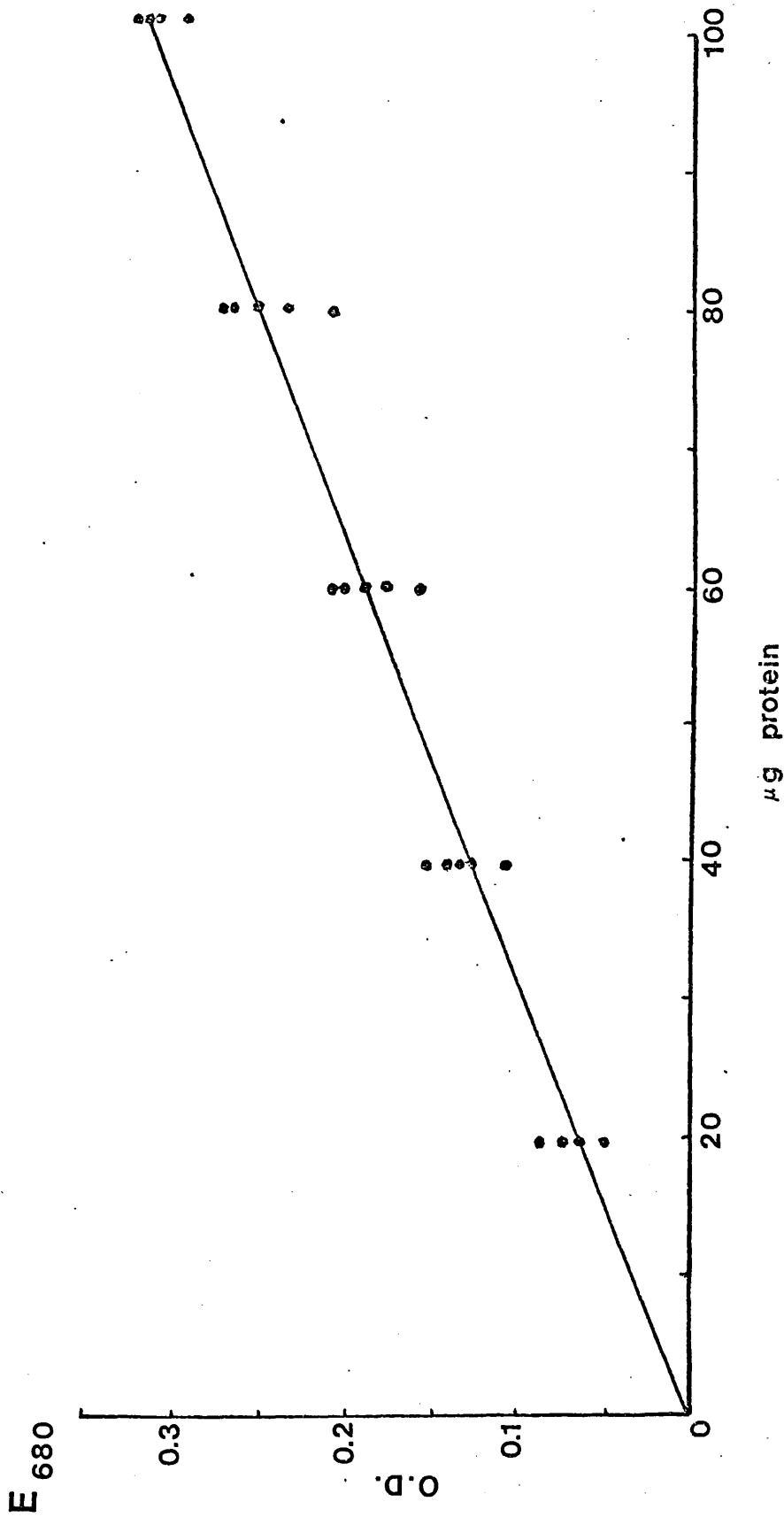
The determination of protein in each preparation was achieved by

a method adapted from Lowry et al. (1951). The solutions involved included:-

- i) 2%  $\text{Na}_2\text{CO}_3$  in 0.1 N-NaOH (20 g  $\text{Na}_2\text{CO}_3$ , anhydrous, added 4 g NaOH per liter of total solution; reagent A).
- ii) 0.5%  $\text{Cu SO}_4 \cdot 5 \text{H}_2\text{O}$  in 1% trisodium citrate.  $2 \text{H}_2\text{O}$  (reagent B).
- iii) 50 parts of A were freshly mixed with one part of B to give reagent C.
- iv) Folin Ciocalteu reagent (as supplied by BDH) was diluted 1:2.3 with distilled water to yield 1 N solution with respect to acid (reagent D).
- v) Protein standards were prepared from a stock solution containing 10 mg/100 ml of bovine serum albumin.

First 4 ml of reagent C were added to sample solutions containing up to 100  $\mu\text{g}$  of protein in a total volume of 0.8 ml, and allowed to stand for 10 min. at room temperature. Then 4 ml of reagent D was added and the mixture shaken rapidly and thoroughly; at least 20 min were allowed for the blue colour to develop, before measuring the absorbance at 680 nm in an MSE Spectro-plus spectrophotometer against a blank without added protein.

A standard curve was prepared using samples from the bovine serum albumin solution (see fig. 2.4). It shows that at 680 nm the optical density increases linearly with protein concentration within the range of 0-100  $\mu\text{g}$  protein.



**Fig. 2.4** Estimation of protein by the method of Lowry et al. (1951).

This standard curve was obtained using known amount of bovine serum albumen (see also text, section 2.6.1). Each sample was read at 680 nm against a reagent blank which contained no protein.

### 2.6.2 Correction for the interfering compounds

Many of the preparations tested for their protein content throughout this work contained various compounds (e.g., indoles & glycerols) which are known to affect the reaction on which the above method is based. For instance, vinblastine (a double indole compound), at a final concentration of 1 mM in 0.8 ml, gives an increase in optical density equivalent to 80  $\mu$ g of protein, but below 0.1 mM its effect was negligible (Tan, L.P. 1975). Glycerol at concentrations between 0.5 M and 3 M gives a linear increase in the colour intensity corresponding to 40-125  $\mu$ g. It was therefore necessary to include the appropriate blank when any of these compounds were present in the protein sample.

### 2.7 Polyacrylamide gel electrophoresis

The purity and molecular weight of tubulin preparations were determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl (Lauryl) sulphate (SDS; Fisons) used to dissociate proteins into their subunit polypeptides. Under these conditions, it was possible to calculate the apparent molecular weights of the individual polypeptides from their mobilities on the basis of a standard 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 Asborn, 1969).

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

It was found that polyacrylamide gel electrophoresis of reduced

carboxymethylated and alkylated preparation 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  $\alpha$  and  $\beta$  subunits according to IUPAC (Webb, 1964; Bryan & Wilson, 1971). Later, it was shown that purified tubulin would also separate into the same two components when electrophoresis was carried out in the presence of 2.8 M urea under various buffer systems (Feit et al., 1971 a; Bryan & Wilson, 1971; Fine, 1971; Lagnado et al. 1972). Under such conditions, it was necessary to reduce carboxymethylate and alkylate the protein in order to bring about the resolution of tubulin into  $\alpha$  and  $\beta$  monomers.

Throughout this work, the two forms of tubulin were separated in a discontinuous TRIS-glycine buffered system modified from Laemmli (1970) in the presence of 4 M urea according to the method described below.

### 2.7.1 Preparation of gels

The 10% gels were prepared from a stock acrylamide solution containing 20 g acrylamide specially purified for electrophoresis (BDH) and 0.15 g of NN'-methylene bisacrylamide (BDH) dissolved in 100 ml of distilled water.

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) 8 M urea) was mixed with 9 ml of stock acrylamide solution. The mixture was then de-aerated for 10 min before adding 2 ml of freshly prepared 1.5% (w/v) aqueous ammonium persulfate. The polymerization was started by the addition of 30-60  $\mu$ l of N, N, N', N'-tetramethylethylenediamine (TEMED, Koch-Light).

The complete mixture was immediately poured into the siliconised tubes, fixed vertically, stoppered at one end with Parafilm, and a drop of water was added to meniscus of the forming gel. Gelling usually occurred within 20 min, and the gels could be used within an hour of preparation.

### 2.7.2 Preparation of protein samples and electrophoresis

Protein samples were mixed with an equal volume of the incubation buffer (0.025 M TRIS-HCl, pH 6.8, 0.8% SDS (w/v), 2% mercaptoethanol, 2 M urea) such that the ratio of SDS to protein on a weight basis was kept between 2-4 and incubated at 100°C for 5 min. The mixture was first allowed to cool at room temperature, then a drop of glycerol-bromophenol blue (saturated aqueous solution) mixture (1:1 (v/v) ) was added to the sample, and aliquots containing 5-100 µg of protein, depending on the purity of the sample, were applied on top of the gels and covered with electrophoresis buffer (see below). 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 mA/gel supplied from a Shandon-Unoplan Type 2541 power pack. When the dye front (Bromophenol Blue, BDH) reached the bottom of the gels (after approximately 3-5 hours), the power supply was cut off and the gels immediately removed from the buffer and released from their glass tubes.

### 2.7.3 Fixing staining and destaining

Proteins in the gel were fixed in 10% (w/v) sulphosalicylic acid (SSA) for at least one hour, and stained in a 0.25% (w/v) solution of Coomassie-Brilliant Blue R 250 (BDH) dissolved in 45 ml of 50% methanol

and 46 ml of glacial acetic acid. For convenience, these two steps were combined by mixing 13.3 parts of 10% SSA with one part of dye solution. Background staining of the gels was removed by washing with several changes of a destaining solution containing 7% glacial acetic acid in 5% aqueous methanol.

#### 2.7.4 Molecular weight determination of polypeptides

By measuring the length of the gel, the distance migrated by the dye front, 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:--

$$\text{Mobility} = \frac{\text{Gel length before fixing}}{\text{Gel length after fixing}} \times \frac{\text{Distance migrated by protein}}{\text{Distance migrated by dye front}}$$

By plotting the logarithm of the molecular weight against the mobility of standard proteins, a linear standard curve was obtained, from which the molecular weight of the unknown protein could be derived (see fig. 2.5). From this standard curve, the molecular weight of  $\alpha$  and  $\beta$  tubulin subunits was found to be 54,000 and 57,000 respectively (see fig. 2.6).

#### 2.7.5 Densitometric scanning of gels

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. 2.6).

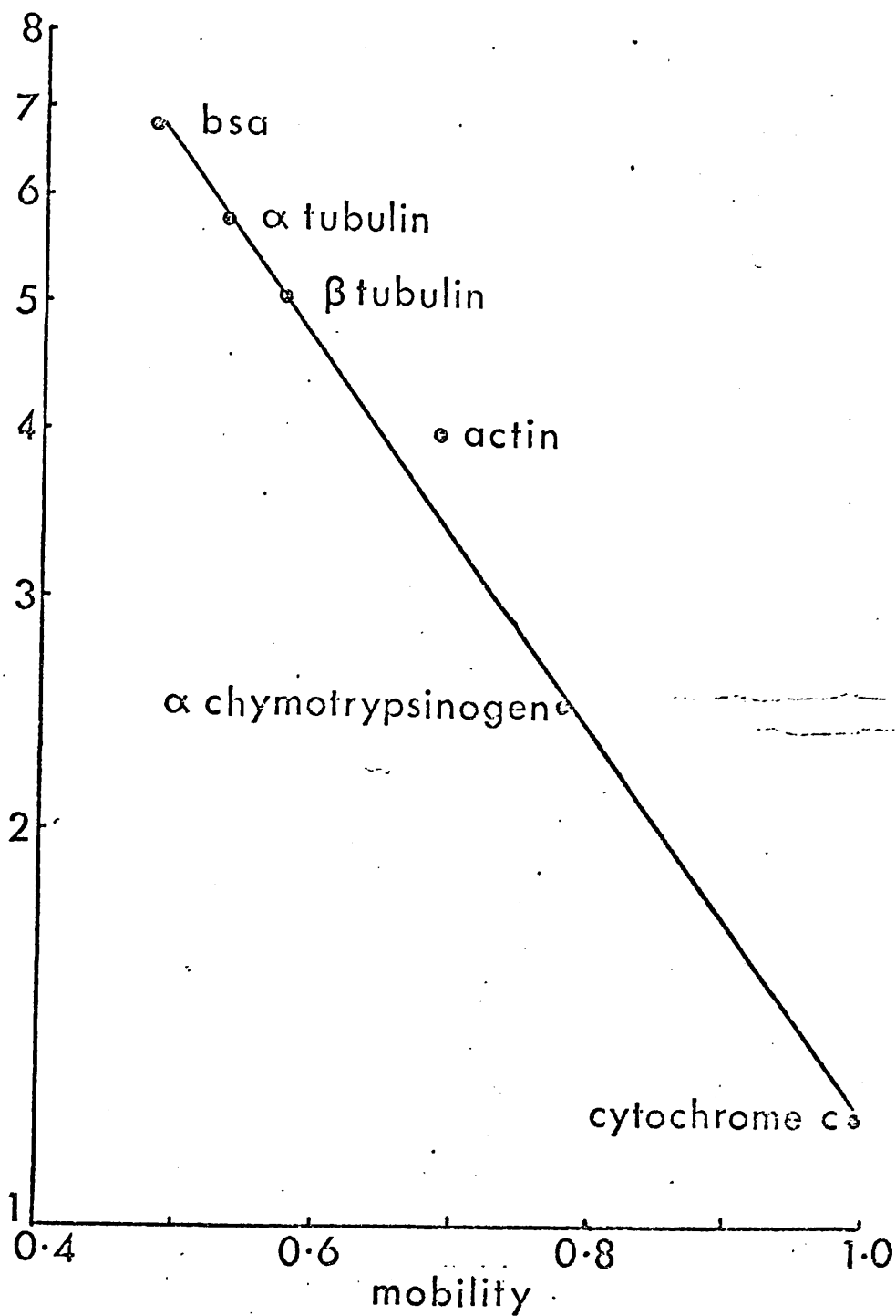


Fig. 2.5 Standard curve for molecular weight determination of purified brain tubulin and other protein components separated by SDS-PAGE (see text, section 2.7.4). Electrophoresis was carried out in tris-glycine buffer system using proteins of known molecular weight.



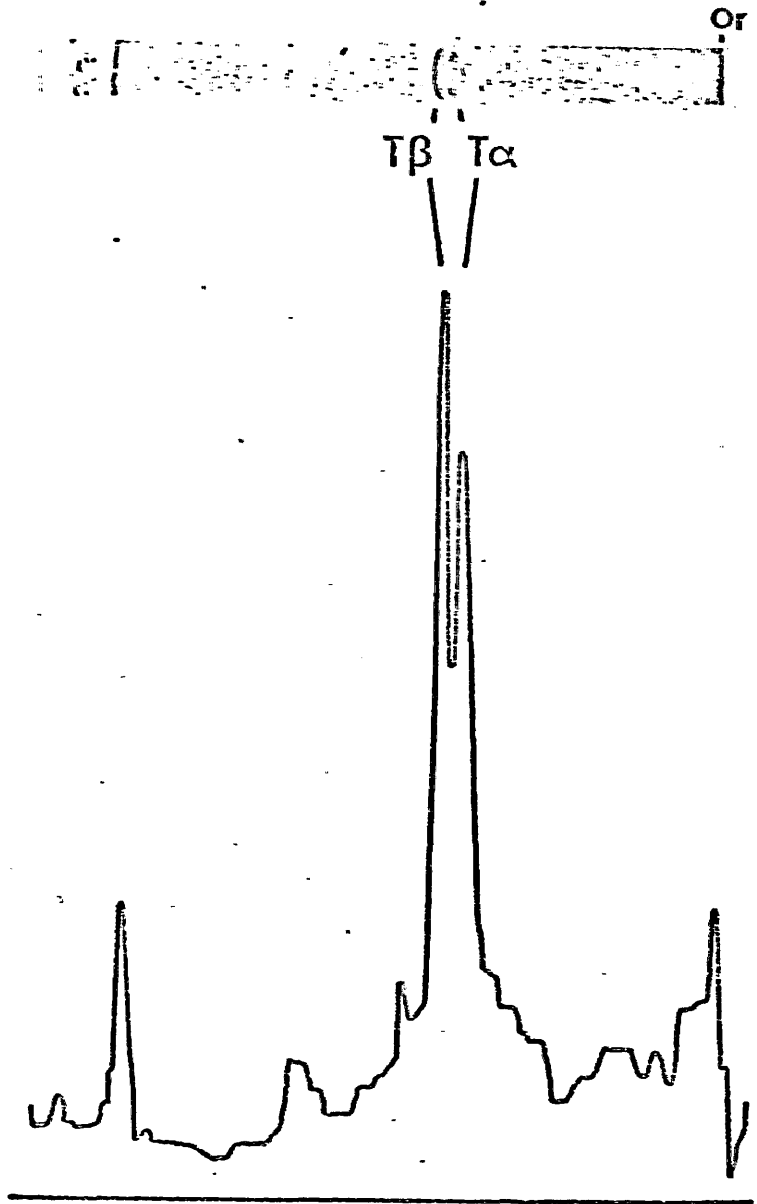


Fig. 2.6 Scanning of an SDS-polyacrylamide gel with purified brain tubulin obtained by one cycle of assembly/disassembly in the presence of glycerol as described in the text, section 2.2.1. Note minor high molecular weight components (HMW) near the origin (Or). For details see also text, section 2.7.5.

## 2.8 Radioactive counting

Radioactive samples were placed in 5 ml of Bray's scintillant (Bray, 1960) and counted in a Tri-Carb Packard 3375 scintillation spectrophotometer. When large volumes of radioactive samples or samples containing compounds with high quenching effect were counted, the scintillant volume was increased to 10 ml.

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

$$\text{Efficiency} = \frac{\text{cpm} \times 10}{\text{dpm}} 10\%$$

It was found that the efficiency for ( $^3\text{H}$ ) was about 48%.

The scintillation mixture used was that of Bray (1960) except that methanol was replaced by ethoxyethanol (EDH) and the amount of naphthalene (BDH), 2,5-Diphenyloxazole (PPO; Fisons) and 1,4-Di-2-(5-phenyloxazolyl)-benzene (POPCP;EDH), were increased to improve the counting efficiency.

### 2.8.1 Preparation of radioactive solutions

The radioactive chemicals obtained from the Radiochemical Centre were adjusted to the required specific activity by the addition of unlabelled compound.

Tritiated colchicine (ring C-methoxyl- $^3\text{H}$ -colchicine) was usually obtained as 250  $\mu\text{Ci}$  in 0.25 ml of ethanol with a specific radioactivity of 4 Ci/m mol. Aliquots of 50  $\mu\text{l}$  of this solution were diluted with an

appropriate volume of unlabelled colchicine solution to give a final concentration of  $50 \mu\text{M}$  and  $0.2 \text{ Ci/m mol}$  final specific activity. This formed the stock solution from which  $50 \mu\text{l}$  were added to the  $0.45 \text{ ml}$  of sample incubation mixture to give a final concentration of  $5 \mu\text{M}$  and  $0.02/\text{m mol}$  specific activity.

The tritiated vinblastine ( $\text{G-}^3\text{H}$  vinblastine sulphate) was supplied as  $250 \mu\text{Ci}$  in  $1.0 \text{ ml}$  of methanol with a specific radioactivity of  $6.3 \text{ Ci/m mol}$ . A volume of  $50 \mu\text{l}$  of this solution was diluted with an appropriate volume of unlabelled vinblastine preparation to give a final concentration of  $50 \mu\text{M}$  and  $0.03 \text{ Ci/m mol}$  final specific activity. From this stock solution,  $50 \mu\text{l}$  were added to the incubation mixture ( $0.45 \text{ ml}$ ) to give a final concentration of  $5 \mu\text{M}$  and  $3 \text{ m Ci/m mol}$  specific activity.

## 2.9 Expression of data

The results of the binding studies (both for colchicine and vinblastine) were expressed either in terms of the number of total bound radioactive counts per minute per fraction (total bd cpm), as specific radioactivities (i.e., number of radioactive counts per minute per mg protein (cpm/mg protein), or as relative specific activities (RSA) as given below:-

$$\text{R.S.A.} = \frac{\% \text{ cpm recovered in fraction}}{\% \text{ protein recovered in fraction}}$$

This enabled better comparisons to be made amongst various fractions. Taking the RSA of the original fraction as 1, values greater than 1 would indicate a relative enrichment of the fraction in actively binding pro-

teins. The means of triplicate determinations for each sample were usually obtained, and formed the basis on which the results are expressed. The values given in the tables throughout this work represent the averages of the different means obtained from separate experiments.

## 2.10 Grid preparation for electron microscopy

Grids were mainly prepared for samples where the possible polymerization of microtubular proteins was investigated.

A drop of the incubated sample was mounted onto a G 400 or G 300 Formvar carbon-coated grid. The excess liquid was drained off carefully with the aid of a filter paper, then a drop of glutaraldehyde was added (and drained off) followed by a wash with a drop of distilled water. The negative staining was achieved by applying 1% uranyl acetate and quickly blotting it off. Since any polymerized microtubules from the synaptosomal fraction (which was the main fraction investigated) were thought to be very unstable, the glutaraldehyde, distilled water, and uranyl acetate solution used were kept at 37°C, during the course of grid preparation in order to preserve optimal conditions for stabilization.

The grids were finally viewed in a Corinth 275 or AEI 6B electron microscope, and selected representative areas were photographed at different magnifications.

Chapter III      The distribution of colchicine and vinblastine receptors  
in subcellular fractions from 1-3 day-old chick brain

3.1      Introduction

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- 3.2.1      Subcellular fractionation
- 3.2.2      Colchicine-binding activity
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Chapter III     The distribution of colchicine and vinblastine receptors  
in subcellular fractions from 1-3 day-old chick brain

3.1     Introduction

Colchicine and the Vinca alkaloids, vinblastine and vincristine, are commonly used as probes for microtubule-dependent processes in various cell types (Oliver et al., 1974; Edelman et al. 1974; Mc Clure, 1972; Nicolson, 1976): both drugs are known to selectively inhibit microtubule assembly in vitro and in vivo and to interact, through separate high affinity binding sites, with tubulin, the subunit protein of microtubules (Wilson et al., 1975; Olmsted & Borisy, 1973).

Early work on the subcellular distribution on colchicine-binding activity in mammalian brain homogenates showed that a significant part of the activity was localised in the synaptosomal fraction. Subfractionation of osmotically lysed synaptosomal preparations from rat brain further indicated that colchicine receptors were present in the synaptosomal fraction as well as in synaptic plasma membranes (see Lagnado et al. 1971 a; Lagnado & Lyons, 1971). The presence of tubulin in isolated nerve ending was recently corroborated by more direct evidence based on chemical analysis of purified synaptic junctional fractions that were enriched in post-synaptic density material (Walters & Matus, 1975), and by electron microscopic observations (Gray, 1975), showing the presence of microtubules in presynaptic elements of cerebral cortex tissue examined in situ.

Taken together, these observations suggest that some of the observed pharmacological effects of antitubulin drugs on synaptic function (Katz, 1972; Hanbauer et al. 1974; Wooten et al. 1975; Poisner, 1973; Sorimachi

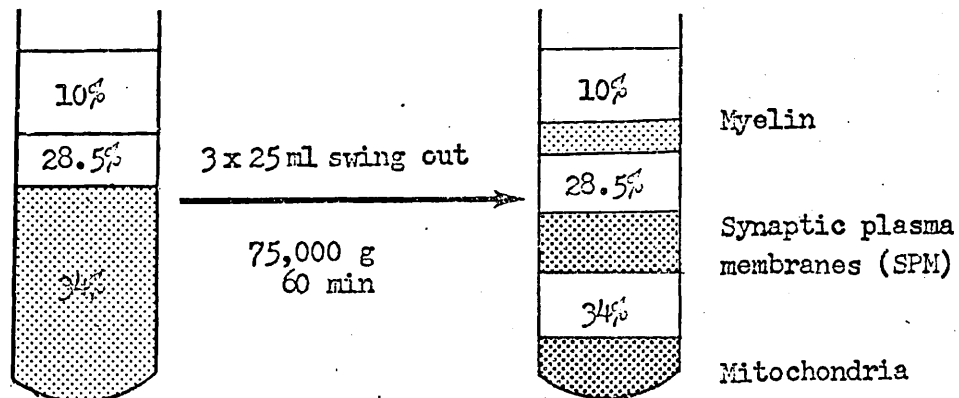
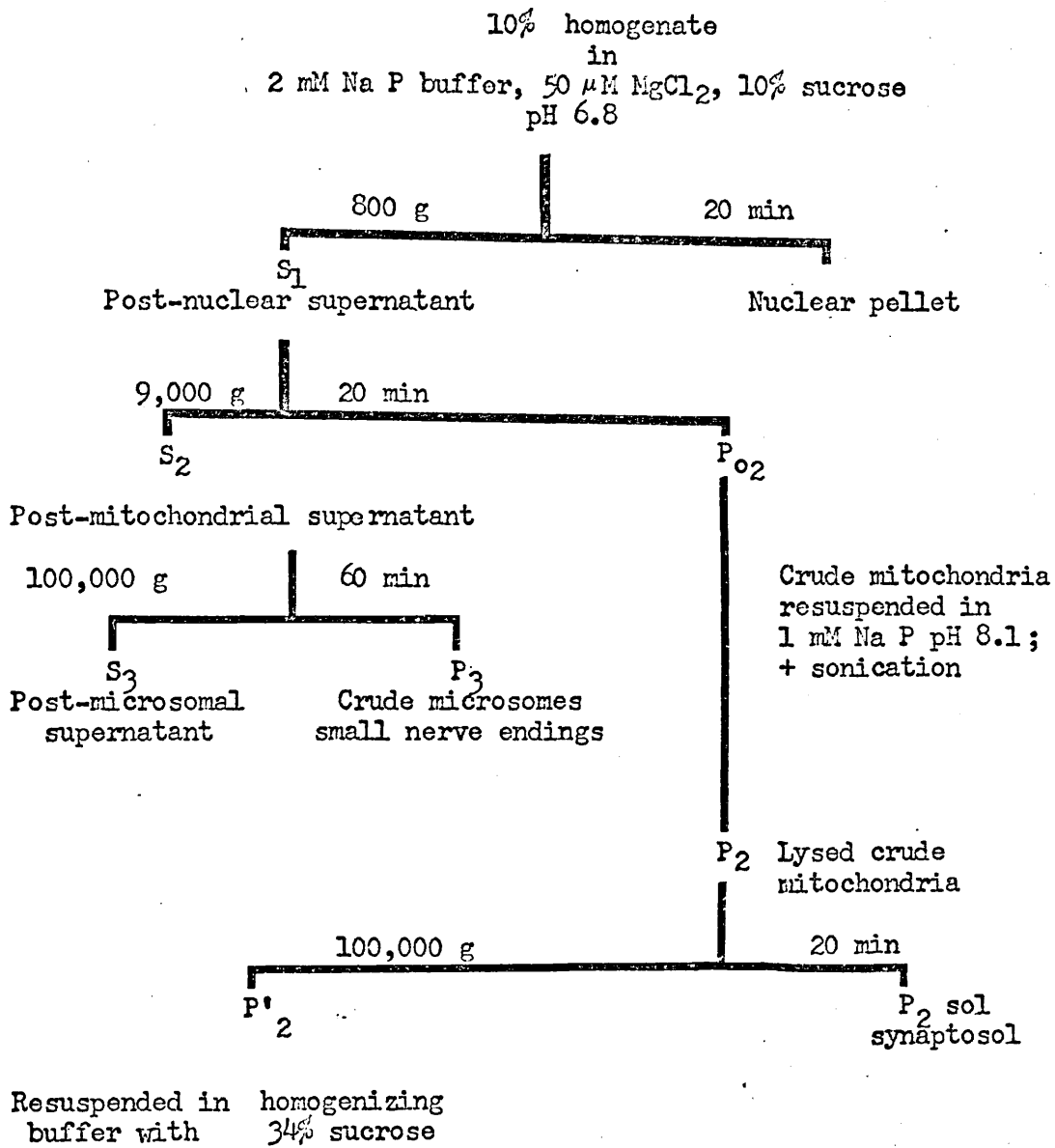
et al. 1973) may be related to a direct interaction of the drugs with tubulin associated with synaptic components.

The main object of the present work was to study the distribution of both colchicine and vinblastine receptors in the soluble and particulate subfractions of chick brain homogenates. For this purpose, chick brain homogenates were prepared and fractionated by the rapid sedimentation-flotation procedure of Jones and Matus (1974), modified in such a way as to yield an additional subsynaptosomal soluble fraction which I will refer to as the synaptosol. The effects of vinblastine on the colchicine-binding activity of individual fractions was also studied, since this drug is known to increase colchicine-binding activity through stabilization of colchicine binding sites against thermal inactivation (see Chapter I). Electron microscopy was used to assess the homogenates of the fraction obtained and limited investigations on the enzymic properties of the fraction were also carried out.

## 3.2 Methods

### 3.2.1 Subcellular fractionation

Whole brains from 1-3 day-old chicks were homogenized and fractionated according to the method of Jones and Matus (1974); except that a phosphate-magnesium buffer (2 mM sodium phosphate, 5 mM  $MgCl_2$ , adjusted to pH 6.8) replaced the Tris-calcium buffer system used by Jones and Matus in the homogenizing medium. This buffer was also incorporated into the sucrose gradient solutions. Lysis of the 'crude mitochondrial pellet' ( $P_{02}$  fraction, see fig. 3.1) was carried out in the absence of sucrose and after adjusting the pH to 8.1. All particulate fractions were resuspended



**Fig. 3.1** Scheme for the subcellular fractionation of chick brains in sodium phosphate buffer, according to the Jones and Matus method. See also text, section 3.2.1.



at 4°C in the phosphate-magnesium buffer (pH 6.8) immediately before assays (see below).

The fractionation procedure illustrated in fig. 3.1 was carried out as follows: usually 5-10 g of brain tissue were homogenized in 9 volumes of buffer and centrifuged at 800 g for 20 min giving a 'crude mitochondrial pellet' (fraction P<sub>2</sub>, fig. 3.1), which formed the starting material for further subfractionation by sucrose gradient centrifugation techniques, and a supernatant which was subjected to high speed centrifugation, 100,000 g for 60 min, to yield the crude microsomal (P<sub>3</sub>) and soluble (S<sub>3</sub>) primary fractions (see fig. 3.1).

The crude mitochondrial pellet (P<sub>2</sub>) was resuspended in 2mM sodium phosphate buffer pH 8.1 (2 ml of buffer per g of wet weight tissue) followed by six strokes in a hand operated homogenizer and kept on ice for 30 min to lyse. Where employed, sonication was effected by an MSE 150 watt ultrasonic desintegrator operated at medium power with optimal amplitude for 3 x 10 secs. At this stage, an additional step was added to the Jones and Matus method and which involved the centrifugation of the lysate, at 100,000 g for 30 min in the cold, in order to extract the synaptosomal (P<sub>2</sub> sol), prior to further subfractionation of the particulate material (Van Leeuwen, 1976). The latter was resuspended in 34% sucrose buffer solution and redistributed onto three 25 ml transparent centrifuge tubes which fitted into the buckets of the 3 x 25 ml swing out rotor (MSE). The suspension was overlaid with 28.5% (w/w) sucrose phase (10.0 ml), which in its turn was overlaid with 10% (w/w) sucrose (5.0 ml) bringing the total final volume to around 20 ml (see fig. 3.1). After centrifugation at 75,000 g for 60 min in an MSE superspee 40, the myelin fraction was recovered from the interface between the 10% and 28.5% sucrose layers.

The synaptic plasma membrane material was recovered from the 28.5% and 34% interface and the mitochondrial fraction was obtained as a pellet (see fig. 3.1).

The contents and distribution of proteins in the different fractions obtained were determined using the Folin Lowry method (see Chapter II, section 2.6).

### 3.2.2 Colchicine-binding activity

Aliquots, containing 200 to 400  $\mu\text{g}$  protein, from the freshly obtained fractions were incubated in a total volume of 0.5 ml in the presence of 10 mM sodium phosphate buffer, containing 5 mM  $\text{MgCl}_2$  (pH 6.8) and 50  $\mu\text{l}$  of an ( $^3\text{H}$ ) colchicine solution (specific activity 0.2 Ci/m mole), to give a final concentration of 5  $\mu\text{M}$  colchicine. When the vinblastine effect on colchicine binding was investigated, the drug was added at a final concentration of 100  $\mu\text{M}$ .

At the end of a 90 min incubation at 37°C, the reaction was stopped on ice and the fractions assayed for binding activity using the DE 81 filter disc assay (see Chapter II, section 2.3.2).

### 3.2.3 Vinblastine-binding activity

This was performed on the same fractions, under the above-mentioned conditions except that the incubation time was reduced to 30 min, since it was shown in preliminary experiments that vinblastine binding remained constant between 10-60 min incubation at 37°C. G  $^3\text{H}$  vinblastine (specific activity 30 Ci/Mole) was used at a final concentration of 5  $\mu\text{M}$ . The binding activity was assayed using the appropriate DE 81 filter disc method (see Chapter II, section 2.4).

3.2.4 Electron microscopy

The main fractions obtained from the sucrose gradients (see fig. 3.1) were examined by electron microscopy. Pellets from these tissues were cut directly (not more than 1 mm cube in size) into the fixative at room temperature.

The fixation (2 hrs) was in 5% glutaraldehyde buffered with disodium hydrogen phosphate and potassium dihydrogen phosphate pH 7.2. Following a thorough washing (2 x 1 hr in buffer only), the tissue was postfixed (1 hr) in 1% osmium tetroxide, buffered with phosphate at pH 7.2 and rinsed twice with distilled water.

The dehydration was performed in an acetone series (30%, 50%, 70%, 90% and 2 x 100%, for 15 min each) followed by 50:50 propylene oxide: acetone and 2 x propylene oxide, also 15 min each.

The samples were then infiltrated with a TAAB resin mixture which was allowed to polymerize in embedding moulds at 60°C for 48 hours.

Sections at 750 - 1000 Å thickness were then cut from each sample using a Huxley ultramicrotome. Such sections were further subjected to lead citrate staining (Reynolds), mounted on 200 F grids and viewed in a Corinth 275 or AEI 6B electron microscope.

Selected representative areas of the grids were finally photographed at different magnifications for each sample.

3.3 Results

3.3.1 Electron micrographs

All three particulate subfractions of the lysed crude mitochondria

(P<sub>2</sub>-lysed) were examined by electron microscopy.

Under such examination, fraction 1, a white band separating between the 10% and the 28.5% sucrose layers, was found to consist mainly of particles (0.8 - 4  $\mu$ m in diameter) surrounded by multilayered membranes which are a characteristic morphological feature of myelin (see fig. 3.2 A and 3.2 B).

Fraction 2, recovered as a grey band at the interfaces of the 28.5% and 34% sucrose layers of the gradient, showed mainly empty membrane-bound particles (0.4 - 0.8  $\mu$ m in diameter, see fig. 3.3 A and 3.3 B), similar in size and appearance to those described as synaptic plasma membranes, SPM, in published micrographs (e.g., Leeuwen et al., 1976; Babitch et al., 1976; Jones, D.H. Ph. D. Thesis, 1976). Occasionally, poorly defined material, resembling synaptic vesicles, can be seen within these particles. Some of these structures were associated with recognisable synaptic clefts (see arrows in fig. 3.3 A & 3.3 B). Mitochondrial or myelin profiles were only rarely seen in this fraction. Also seen alongside these membrane-bound particles were other smaller dense particles similar to those observed by Jones and Matus (1974) (see dp in fig. 3.3 A & 3.3 B). From this description, it is concluded that this fraction consisted mainly of SPM elements.

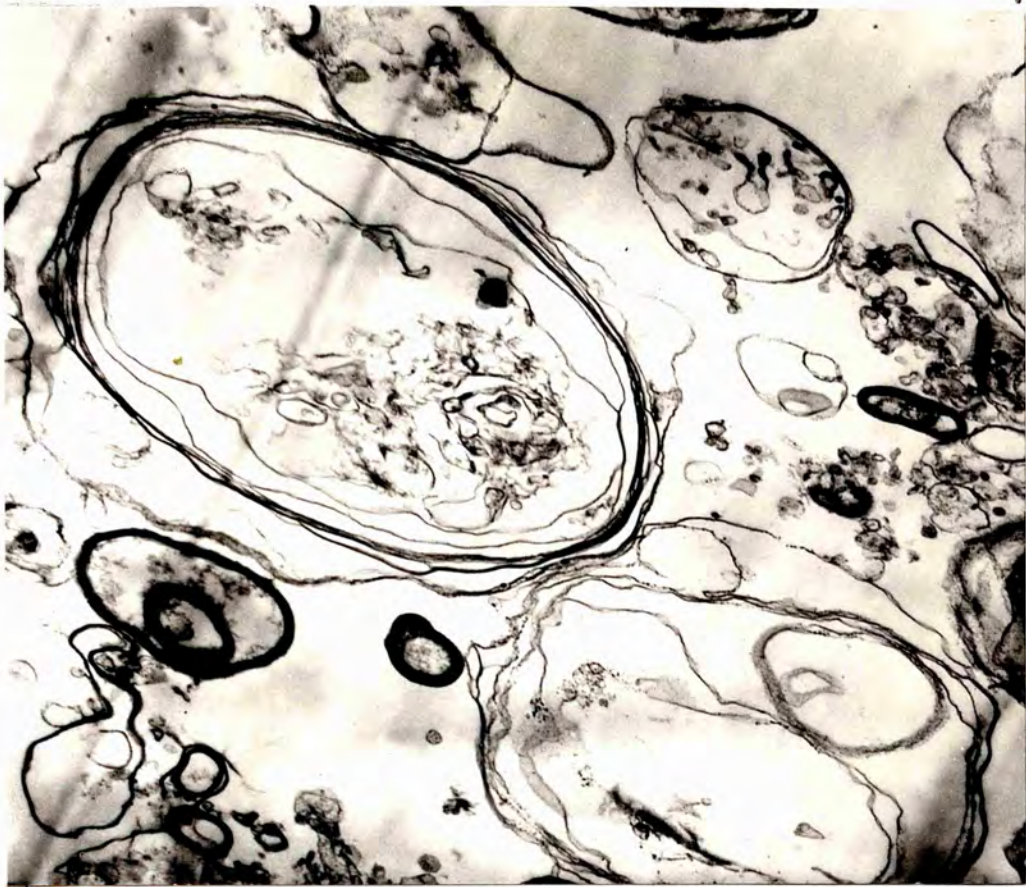
Fraction 3, the brown pellet from the gradient, appeared to consist mainly of mitochondria in which the internal cristae were sometimes clearly apparent (see fig. 3.4 A & 3.4 B).

Thus, electron microscopy indicates that the myelin, SPM and mitochondrial elements were fairly well separated on the basis of the sucrose gradient centrifugation step, when the Jones and Matus method was applied to the crude mitochondrial fraction derived from chick orain homogenates

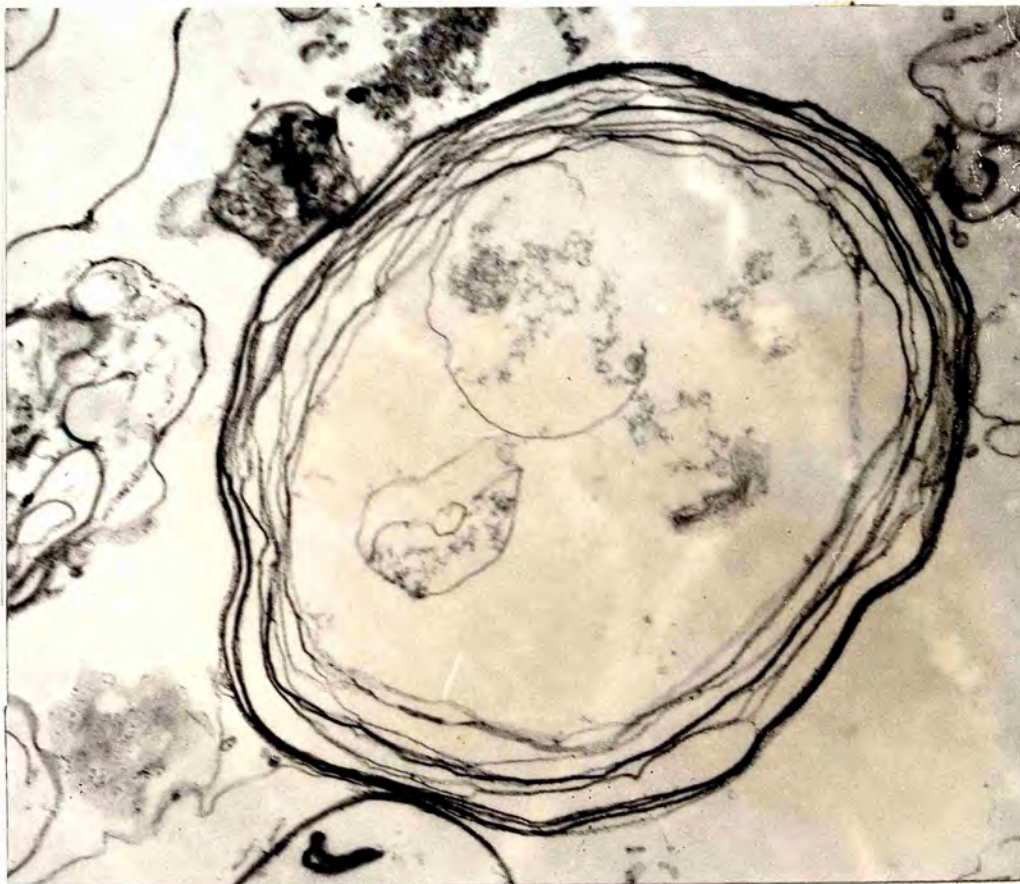


Fig. 3.2 A            Random field of myelin fraction showing  
multilayered-membrane particles (0.8 - 4  $\mu$ m).  
Note small vesicular structures within each  
particle. See also text section 3.3.1.  
Magnification = 12,250.

Fig. 3.2 B            Representative myelin particle. Note the  
multilayered membrane. Magnification = 22,500.



Fi 3.2 A



Fi 3.2 B



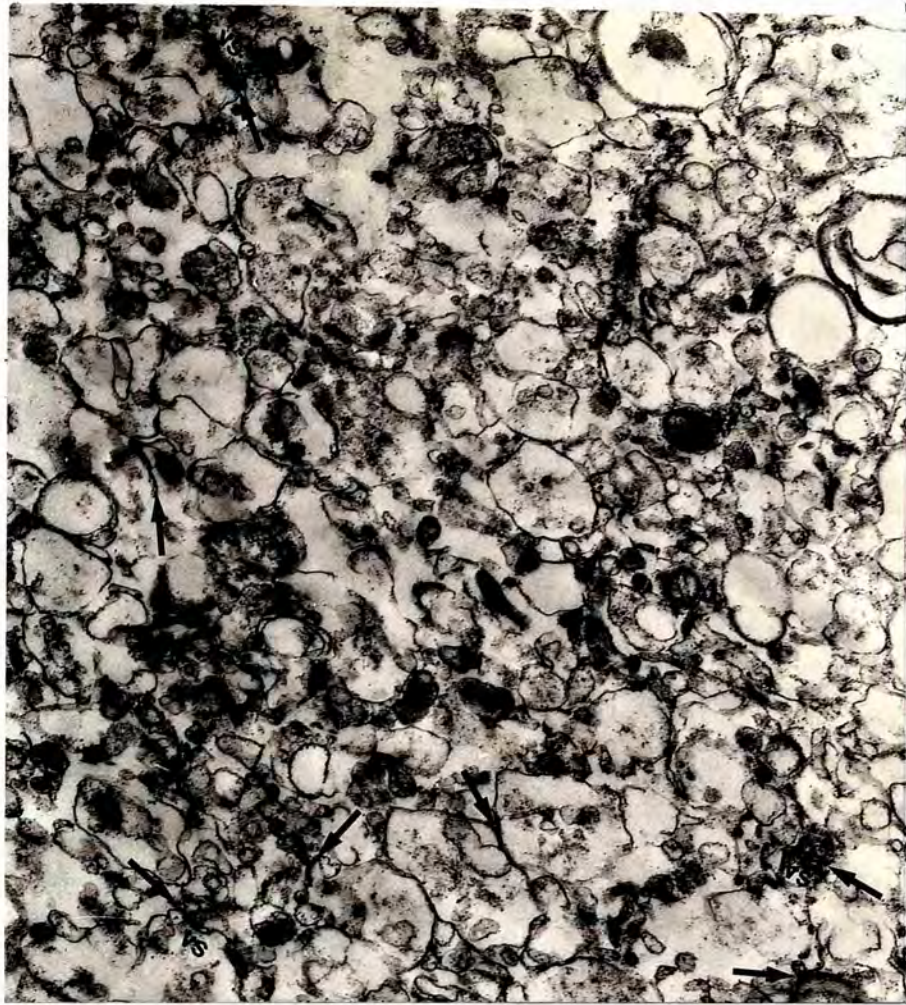


Fig. 3.3 A

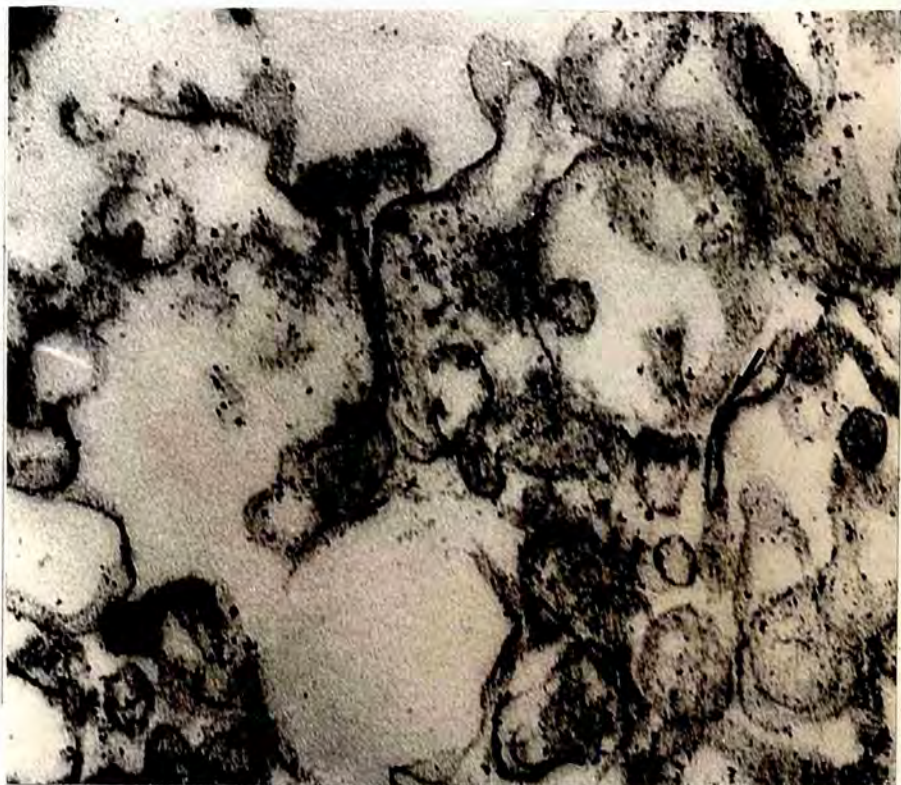
Random field of SPM fraction showing empty membrane-bound particles (0.4 - 0.8  $\mu\text{m}$ ). Many synaptic junctions are recognisable (see arrows). Note the poor presence of synaptic vesicles (marked vs). See also details in text section 3.3.1. Magnification = 27,500.

Fig. 3.3 B

SPM fraction showing representative synaptic junctions (see arrows).  
Magnification = 110,000.



Fi 3.3 A



Fi 3.3 B



Fig. 3.4 A

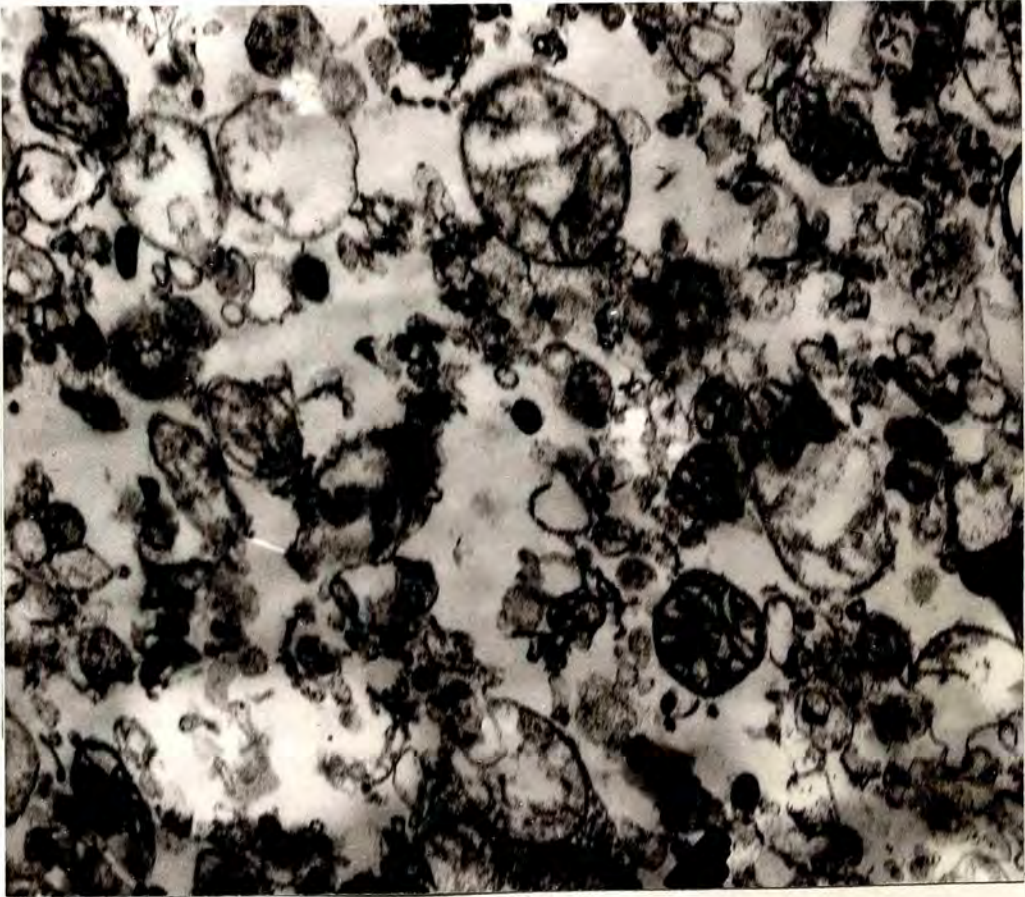
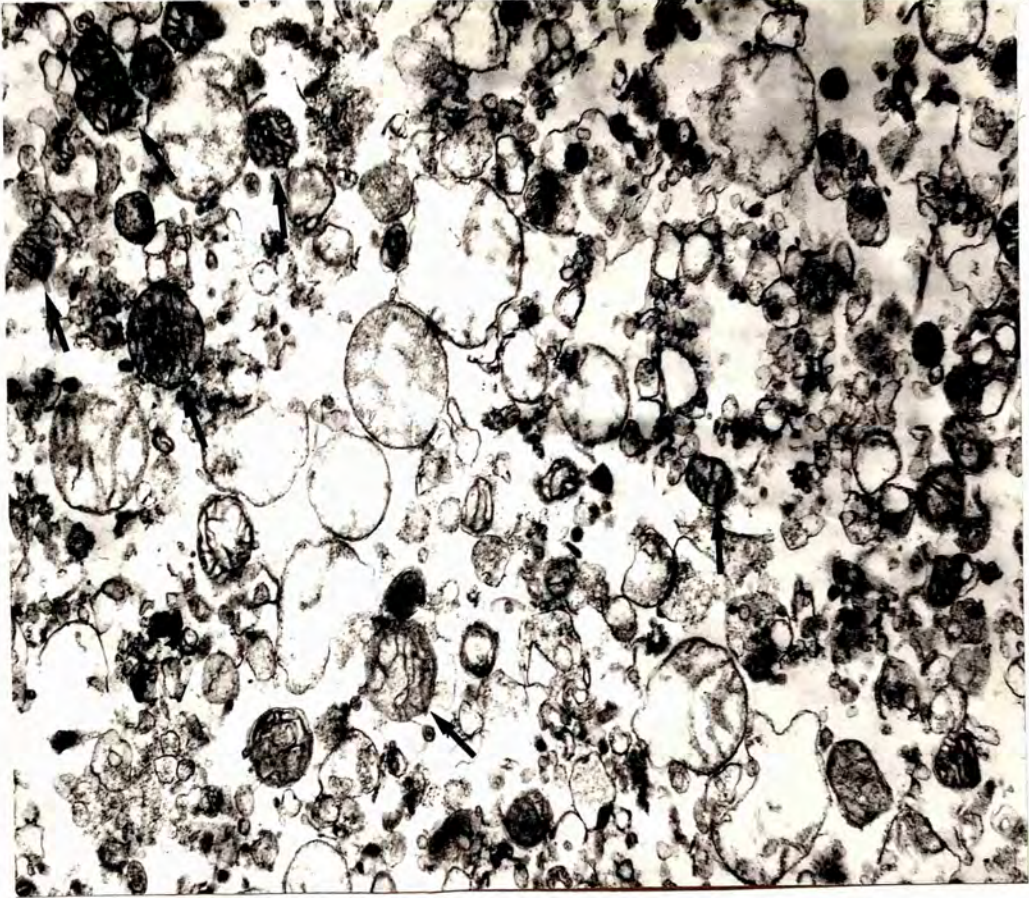
Random field of mitochondrial fraction.

Some of the mitochondria show clearly the internal cristae structures (see arrows). See also text, section 3.3.1. Magnification = 16,000.

Fig. 3.4 B

Random field of the mitochondrial fraction at a higher magnification (22,500).





as opposed to rat brain.

### 3.3.2 Colchicine-binding activity

The results in table 3.1 show that 20% of the colchicine-binding activity in chick brain postnuclear supernatant ( $S_1$ ) was recovered in the crude mitochondrial fraction ( $P_2$ ), the remainder (80%) being more or less equally distributed between the crude microsomal ( $P_3$ ) and soluble ( $S_3$ ) fractions, both derived from  $S_2$  (see table 3.1). It should be noted however that, under the present conditions, fraction  $P_3$  probably also contains small nerve ending particles (see Lapetina et al., 1967). A slight enrichment could be noticed in the soluble subfractions  $S_2$  and  $S_3$  (see also fig. 3.5 A & 3.5 B) with  $S_3$  showing the highest specific activity of all the primary subfractions investigated (i.e.  $S_2$ ,  $P_2$ ,  $S_3$ ,  $P_3$ ; see also fig. 3.9, light columns).

Amongst the subfractions of the osmotically lysed  $P_2$  fraction, marked differences were observed in the relative distribution of binding activities. It is clear from the PSA values shown in table 3.1 and fig. 3.8 that the  $P_2$  solution 'synaptosol' and synaptic plasma membrane (SPM) fractions were enriched in colchicine-binding activity. It is noteworthy that the distribution pattern of the binding activity amongst the subfractions of chick brain homogenates reported here is very similar to that previously found for rat brain homogenates fractionated by entirely different procedure (see Lagnado et al., 1971a).

### 3.3.3 Effects of vinblastine on colchicine-binding activity

The presence of vinblastine (100  $\mu$ M, final concentration) in the reaction mixture, stabilized the colchicine-binding activity in all the



Table 3.1 Distribution of colchicine-binding activities, in the presence and absence of vinblastine (Vb),

in subfractions of chick brain postnuclear supernatant.

Results shown are means of triplicate determinations from three separate experiments (<10% difference between experiments). Values in the first two columns are given as a percentage of protein or binding activity recovered from mother fractions: (S<sub>1</sub>), postnuclear supernatant; (S<sub>2</sub>), postmitochondrial supernatant; (P<sub>02</sub>), lysed crude mitochondrial preparation. RSA, relative specific radioactivity, = % bound c.p.m. recovered / % protein recovered. Binding activities were assayed radiometrically as described in Chapter II, section 2.3.2. For more details see also text, sections 3.3.2 and 3.3.3.



Colchicine-binding activity

Primary fraction	Sub fraction	Description	Protein %	Bound cpm distribution %	Colchicine-binding activity			
					RSA without Vb	$10^3$ x specific radioactivity with Vb	$10^3$ x specific radioactivity R.S.A (+ Vb)	
S <sub>1</sub>	S <sub>2</sub>	Post mitochondrial supernatant	75	81	1.02	20.0	29.6	1.56
	P <sub>02</sub>	Crude mitochondria (lysed)	21	19	0.91	21.7	28.4	1.31
S <sub>2</sub>	S <sub>3</sub>	Post microsomal supernatant	43	54	1.26	28.0	43.1	1.55
	P <sub>3</sub>	Crude microsomes + small nerve endings	57	46	0.81	20.0	27.4	1.37
P <sub>02</sub>	P <sub>2</sub> sol	Synaptosol	23	33	1.43	36.0	56.4	1.57
	P <sub>2</sub> H <sub>2</sub> O	My	5	4	0.80	28.0	24.4	0.87
		SPM	19	23	1.22	36.3	50.4	1.39
	Mit	Mitochondria	53	40	0.76	29.8	32.2	1.08

Table 3.1



Fig. 3.5 Relative distribution of the colchicine-binding activity in primary subcellular fractions of chick brain (see text, section 3.3.2).

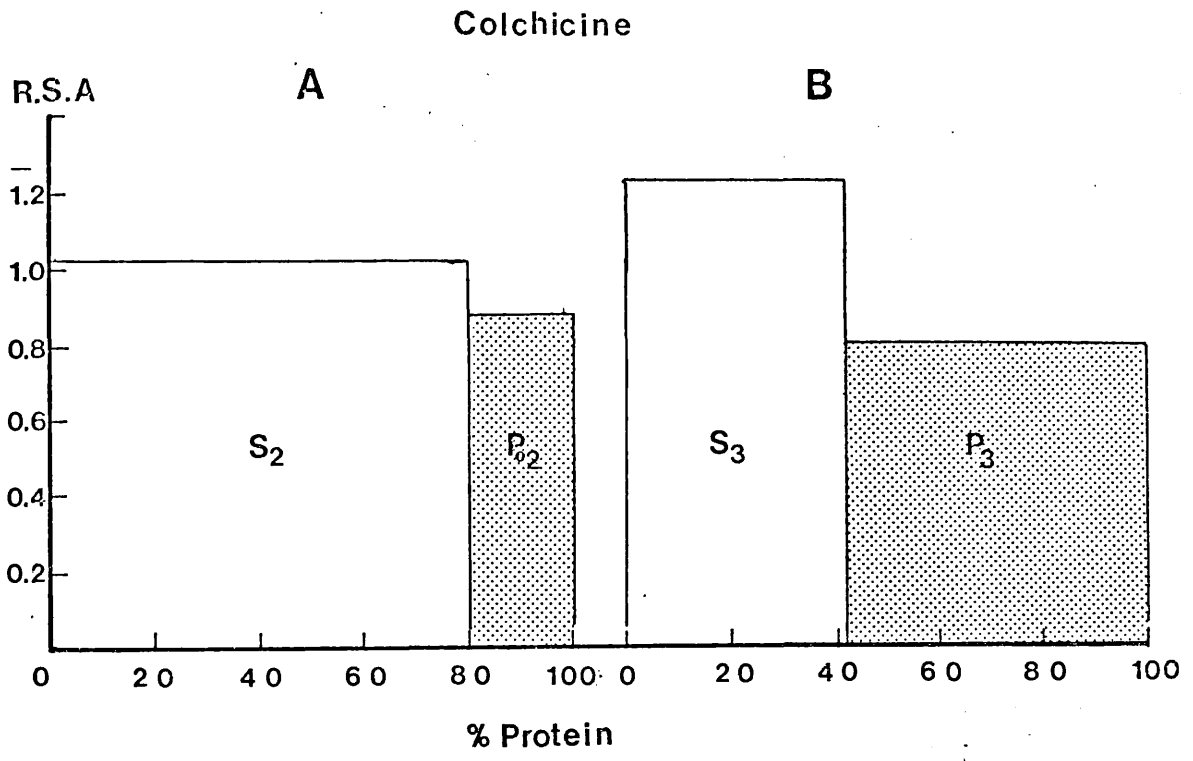
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Subcellular fractionation was carried out using a modification of the method of Jones and Matus to yield fractions: (S<sub>2</sub>), post-mitochondrial supernatant; (P<sub>02</sub>), lysed crude mitochondria; (S<sub>3</sub>), postmicrosomal supernatant; (P<sub>3</sub>), crude microsomes + small nerve-endings. RSA = % bound c.p.m. recovered/% protein recovered.

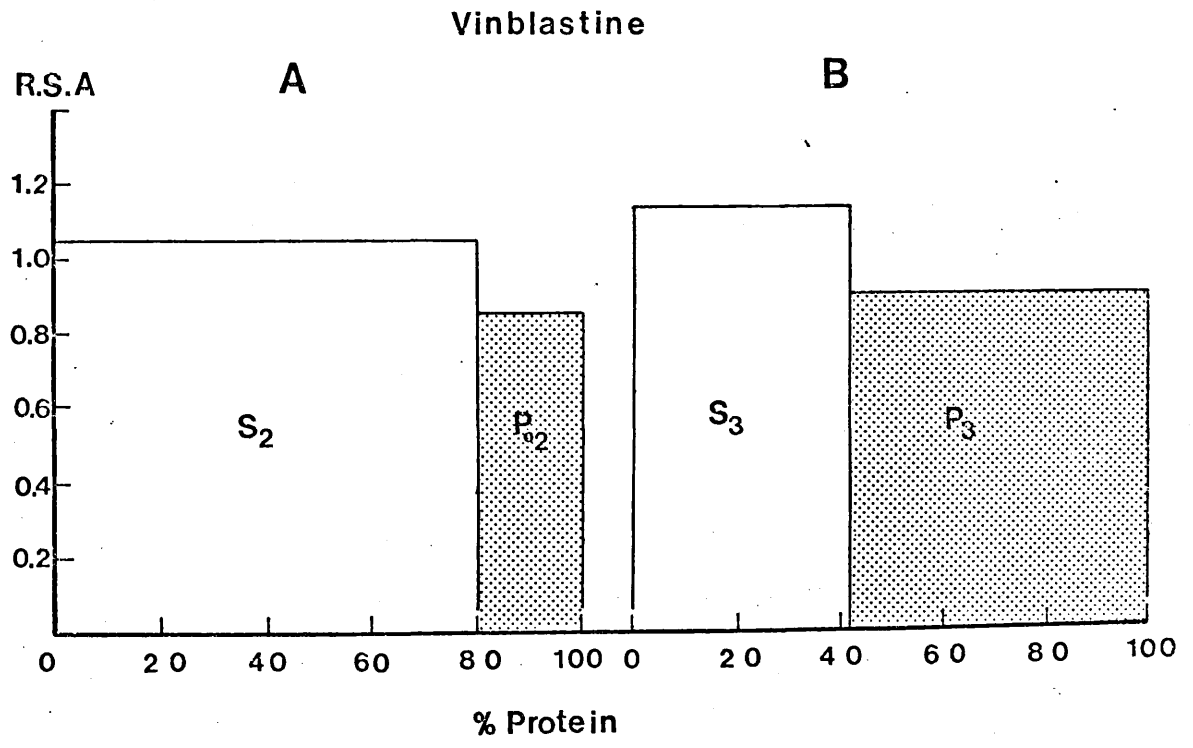
Fig. 3.6 Relative distribution of the vinblastine-binding activity in primary subcellular fractions of chick brain (see text, section 3.3.4).

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Subcellular fractionation was carried out using a modification of the method of Jones and Matus to yield fractions: (S<sub>2</sub>), post-mitochondrial supernatant; (P<sub>02</sub>), lysed crude mitochondria; (S<sub>3</sub>), postmicrosomal supernatant; (P<sub>3</sub>), crude microsomes + small nerve-endings. RSA = % bound c.p.m. recovered/% protein recovered.



**Fig. 3.5**



**Fig. 3.6**



Fig. 3.7 Relative distribution of the vinblastine-binding activity in subfractions of crude lysed mitochondria from chick brain (see text, section 3.3.4).

Subfractions were separated by the sedimentation-floatation gradient technique (Jones & Matus), and included subfractions: (P<sub>2</sub>sol), synaptosol; (My), myelin; (SPM), synaptic plasma membranes; (Mit), mitochondria. RSA = % bound c.p.m. recovered/% protein recovered.

Fig. 3.8 Relative distribution of the colchicine-binding activity in subfractions of crude lysed mitochondria from chick brain (see text, section 3.3.4).

Subfractions were separated by the sedimentation-floatation gradient technique (Jones & Matus), and included subfractions: (P<sub>2</sub>sol), synaptosol; (My), myelin; (SPM), synaptic plasma membranes; (Mit), mitochondria. RSA = % bound c.p.m. recovered/% protein recovered.

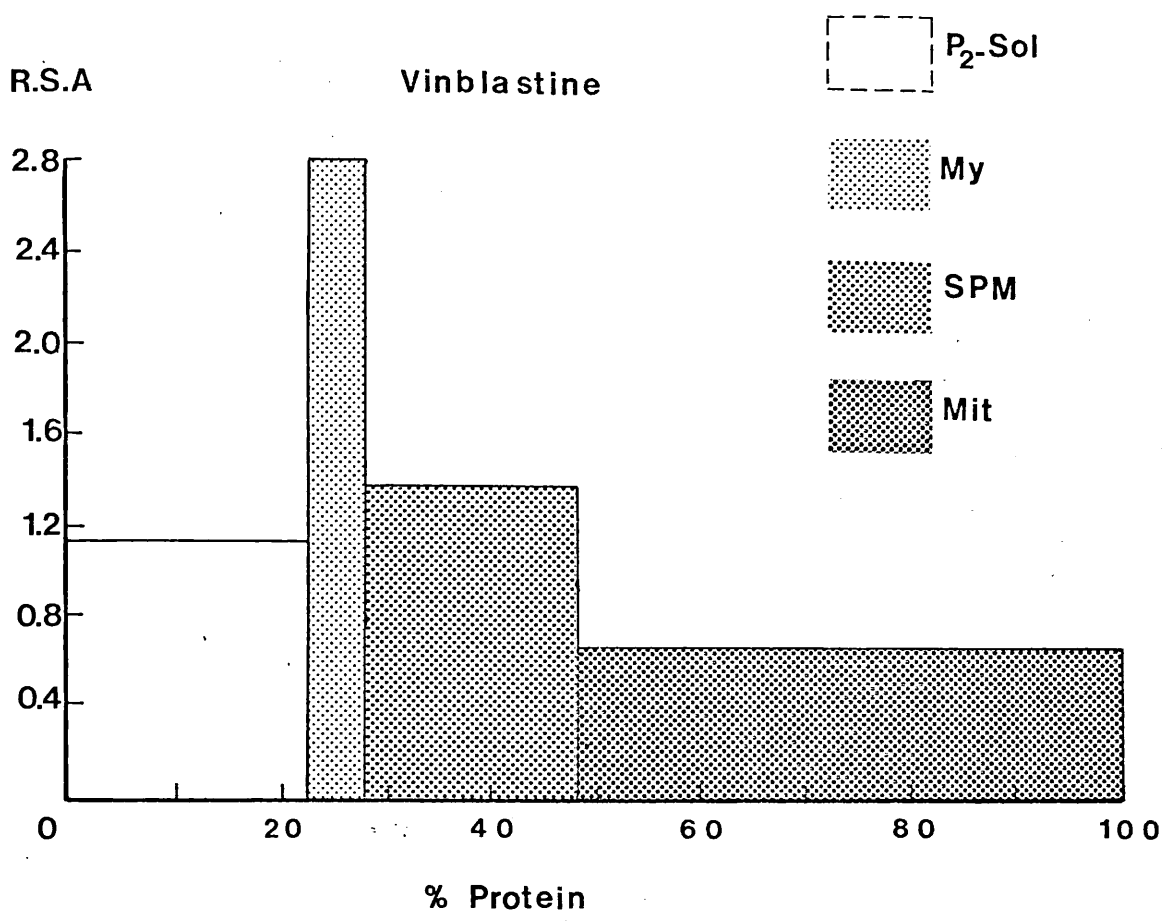


Fig.3.7

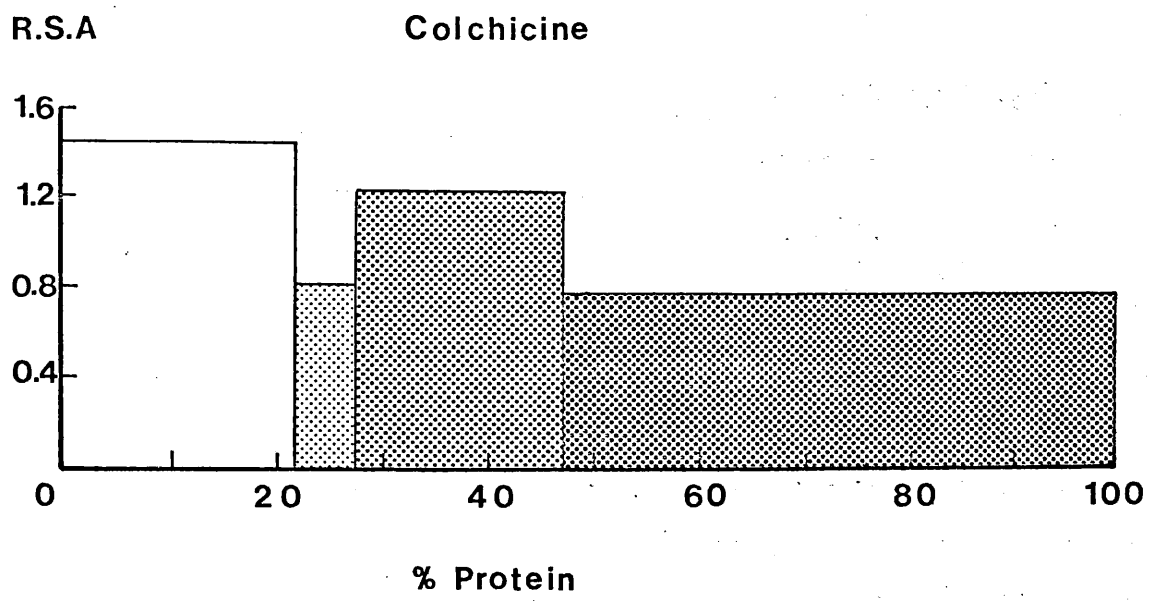


Fig.3.8



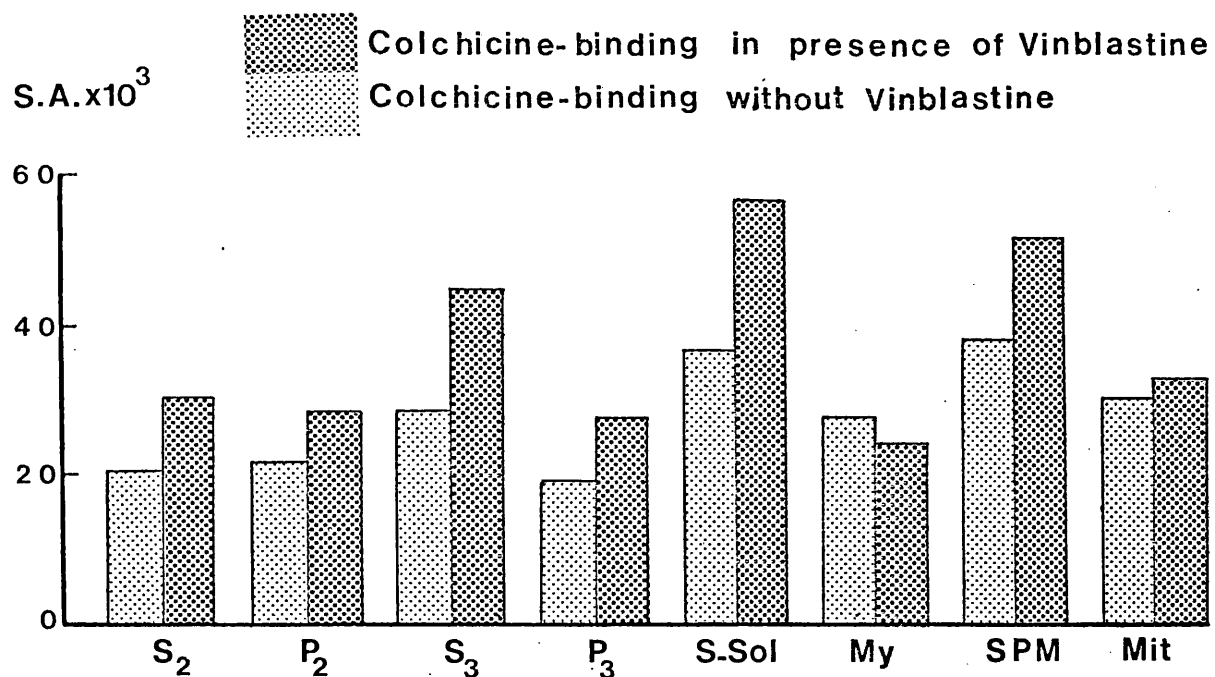


Fig. 3.9 Distribution of colchicine-binding activity, in the absence and presence of vinblastine, in primary and lysed crude mitochondrial subfractions of chick brain.

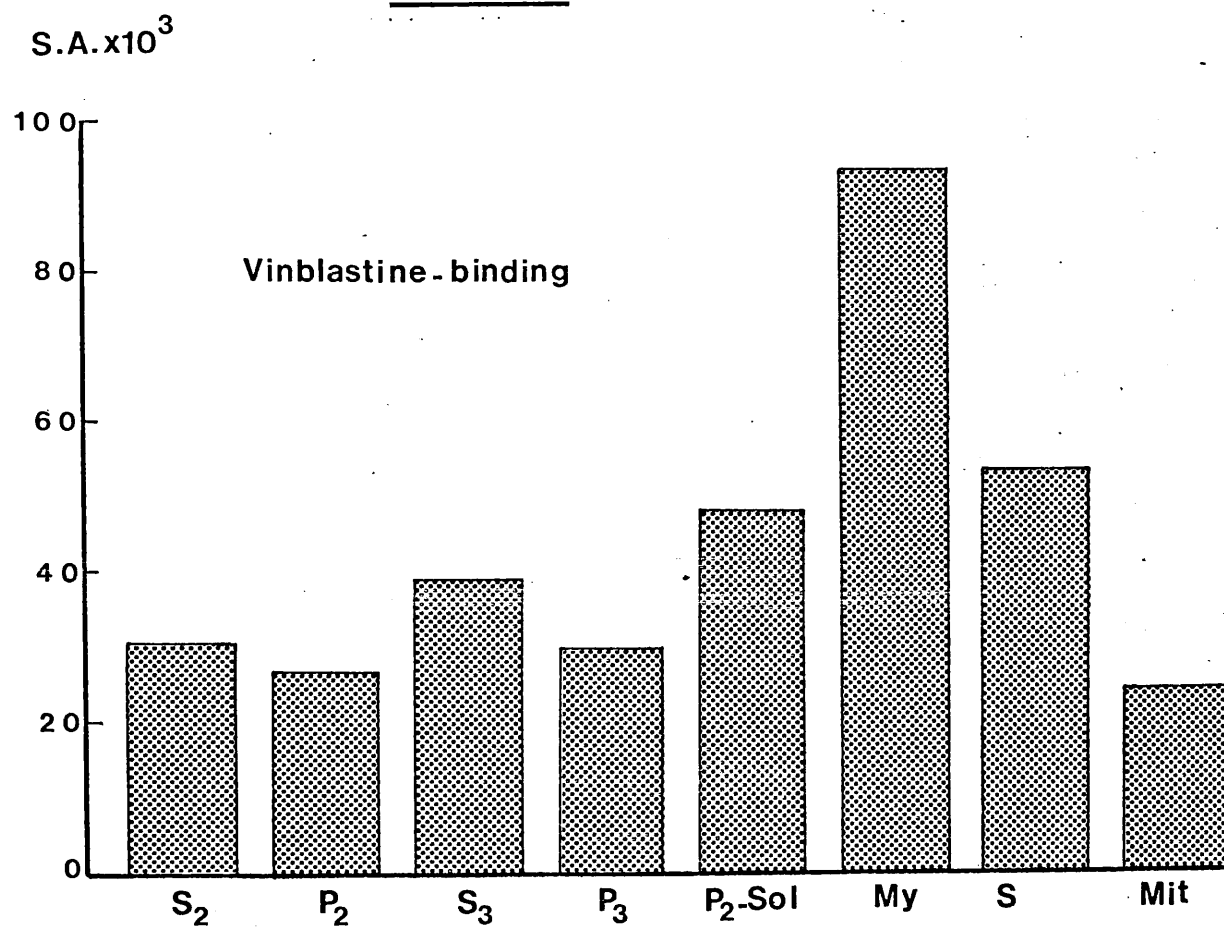
Light shaded columns = colchicine-binding activity in absence of vinblastine; dark shaded columns = colchicine-binding activity in presence of vinblastine (100 M, final concentration). For subfractions description see table 3.1 and text, sections 3.3.2 and 3.3.3. S.A. , specific radioactivity, represents bound c.p.m./mg of protein.

Fig. 3.10 Distribution of vinblastine-binding activity in primary and lysed crude mitochondrial subfractions of chick brain.

For subfractions description see table 3.2 and text, section 3.3.4. S.A., specific radioactivity, represents bound c.p.m./mg of protein.



**Fig.3.9**



**Fig.3.10**



Table 3.2 Distribution of vinblastine-binding activities in subfractions of chick brain postnuclear supernatant.

Results shown are means of triplicate determinations from two separate experiments (< 10% difference between experiments). Values in the first two columns are given as a percentage of protein or binding activity recovered from mother fractions: (S<sub>1</sub>), postnuclear supernatant; (S<sub>2</sub>), postmitochondrial supernatant; (P<sub>02</sub>), lysed crude mitochondrial preparation. RSA, relative specific radioactivity, = % bound c.p.m. recovered/% protein recovered. Binding activities were assayed radiometrically as described in Chapter II, section 2.4. For more details see text, section 3.3.4.

Vinblastine-binding activity

Primary fraction	Sub fraction	Description	Protein %	Bound cpm distribution %	Vinblastine-binding activity	
					RSA	$10^3 \times$ specific radioactivity
S <sub>1</sub>	S <sub>2</sub>	Post mitochondrial supernatant	79	82	1.04	30.6
	P <sub>2</sub>	Crude mitochondria (lysed)	21	18	0.86	26.7
S <sub>2</sub>	S <sub>3</sub>	Post microsomal supernatant	43	48	1.12	36.8
	P <sub>3</sub>	Crude microsomes + small nerve endings	57	52	0.91	29.8
P <sub>02</sub>	P <sub>2</sub> sol	Synaptosol	23	25	1.09	49.0
	P <sub>2</sub> H <sub>2</sub> O	Myelin (see text also)	5	14	2.80	93.3
		Synaptic plasma membranes	19	26	1.36	52.8
		Mitochondria	53	35	0.66	24.5

Table 3.2

primary fractions and all but one of the lysed mitochondrial subfractions (see fig. 3.9, dark columns). The myelin subfraction was the only one where vinblastine seemed to act as an inhibiting factor on the colchicine binding sites (see SA columns in table 3.1). In general, the increase in specific activities due to stabilization was more pronounced in the soluble fractions (1.6 x the original SA) than in the particulates (1.3 x the original SA). In the case of the mitochondrial fraction, hardly any effect of vinblastine was observed.

From these results, it can be inferred that the characteristic stimulatory effects of colchicine binding reflect the presence, in various fraction of tubulin or tubulin-like protein sharing a property characteristic of soluble tubulin where the effects of vinblastine were first observed (see Chapter I, and present Chapter, section 3.4). Conversely on the same basis, it can be inferred that the colchicine-binding activity, that was associated with the myelin and mitochondria subfractions, may be due to non-tubulin binding elements. One possibility would be that vinblastine interacts with components, in the myelin and mitochondria fraction, that are not related to tubulin (i.e., to CB sites). For this reason, separate experiments were undertaken to determine the subcellular distribution of vinblastine binding sites in chick brain homogenates and the extent to which this could be correlated with the distribution of CB activity.

#### 3.3.4. Vinblastine-binding activity

The results in table 3.2 show that the distribution of vinblastine-binding activities, amongst the primary fractions, is similar to that of colchicine, with the postmicrosomal supernatant ( $S_3$ ) showing again the highest specific activity (see also fig. 3.10). A slight enrichment of all

the soluble primary fractions was also observed in this case (see fig. 3.6 A & 3.6 B).

However, the subfractions derived from the lysed crude mitochondrial fraction behaved differently in the present case. The vinblastine binding sites were concentrated primarily in the myelin fraction (My) when the activity was expressed on a protein basis (see table 3.2, fig. 3.7). It should be noted that the 'myelin' fraction also contains light membranes, whose origin has not been determined. In addition, the fraction containing synaptic plasma membranes (SPM) was also enriched in vinblastine-binding activity, while little enrichment was seen in the synaptosol fraction as compared to that found for colchicine binding (see table 3.2, fig. 3.7 & 3.8).

#### 3.4 Discussion and conclusions

It is clear from the above results that the distribution of colchicine and vinblastine receptors in subfractions of the lysed crude mitochondrial fraction of chick brains ( $P_2$ ) is not strictly parallel: the main contrast being the high concentration of vinblastine binding sites in the crude myelin fraction. It may be interesting, in this connection, that while vinblastine stabilized colchicine-binding activity in the synaptosol ( $P_2$  sol) and synaptic plasma membranes (SPM) fractions, colchicine binding in the myelin fraction was consistently inhibited by about 20% in the presence of 100 M vinblastine.

Furthermore, this observed selective enrichment of vinblastine binding-activity in the crude myelin fraction may reflect the presence in this material of non-tubulin binding sites for vinblastine (see also Wilson et al., 1970), the nature of which remains to be clarified. It is suggested that some of the differences observed in the pharmacological effects of

colchicine and vinblastine on synaptic activity (see e.g., Katz, L., 1972) may be related to differences in specificity between the two drugs.

One obvious problem connected with the interpretation of the distribution studies described in this Chapter relates to the identification of the fractions obtained by the centrifugation scheme employed, since this was originally devised for rat brain (Jones and Matus). The electron microscopic evidence presented seems to correlate reasonably well with that presented by these two authors for rat brain. However, further experiments carried out in collaboration with M.K. Johnson (MRC Toxicology Unit, Carshalton) indicate that while the SPM fraction was clearly enriched in acetyl cholinesterase (AChE) activity, as compared to the myelin and mitochondrial fraction, the distribution of other plasma membrane marker enzymes indicated substantial contamination of the mitochondrial fraction with (synaptic) plasma membrane components. For this reason, it was decided to carry out further experiments with view to achieving a better resolution of the heavier membrane components present in the crude mitochondrial fraction. At the time these experiments were planned, Babitch et al., (1976) reported that the sedimentation rates of chick brain synaptosomal membranes were higher than for the corresponding membranes in the rat brain. This difference would explain anomalous distribution of plasma membrane marker enzymes, referred to above, during the fractionation of chick brain.

At about this time, a detailed study on the "Isolation and partial characterization of chick brain synaptic plasma membranes" was published by Leeuwen et al. (1976) using a modification of the floatation-sedimentation centrifugation technique of Jones and Matus. In this method, the sucrose gradient step, in particular, was altered to enable a clear cut resolution of mitochondria from other membrane components during the



purification of synaptic membranes. This procedure was used to further study the subcellular distribution of colchicine-binding activity in chick brain.

Finally, it is interesting to note that the synaptosomal fraction of chick brain, like that of rat brain (see also section 3.1), was enriched in colchicine-binding activity. The possibility that the tubulin, present in synaptosomes, originated from microtubular structures in nerve endings, such as were described by Gray (1975) will be the subject of further work to be described later (Chapter IV to VI).

Chapter IV      Preliminary attempts to polymerize microtubular protein  
present in synaptosome soluble extracts (synaptosol)

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- 4.2.2      Colchicine-binding activity
- 4.2.3      Gel electrophoresis
- 4.2.4      Polymerization
- 4.2.5      Electron microscopy
- 4.2.6      Protein determination

4.3      Results

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Chapter IV    Preliminary attempts to polymerize microtubular protein present in synaptosome soluble extracts (synaptosol)

4.1    Introduction

The presence of labile microtubules in nerve cell processes is well established and can be correlated with the presence, in crude high-speed extracts of brain, of a pool of in vitro polymerisable microtubule subunit protein (tubulin) exhibiting high affinity binding sites for colchicine and for Vinca alkaloids such as vinblastine and vincristine (see e.g., Snyder and Mc Intosh, 1976; Samson, 1976; this thesis Chapter I). Both types of drugs have been shown to interrupt synaptic functions indirectly, through inhibition of axonal flow (Bank, 1976; McClure, 1972), or possibly through direct effects at the synapse (Katz, 1972; Sorimachi et al. 1973; Wooten et al. 1975).

An obvious possible site of action of these drugs in synapses would be microtubular protein itself. Indeed, as was shown in the previous Chapter (section 3.3), synaptosomes, together with microsomes, accounted for most of the colchicine-binding activity that occurred in the particulate fractions of chick brain homogenate, similarly to what has been observed for rat brain in earlier work (see Lagnado et al. 1971; Feit et al. 1971). Furthermore, it was found that one-third to half of the binding in the synaptosome fraction was recovered in the soluble subfraction (= synaptosol or P<sub>2</sub>-sol) obtained after hypotonic lysis (see Chapter III, section 3.3). It was also shown that the addition of Vinca alkaloids (10-50  $\mu$  M) to synaptosol extracts induces the precipitation of protein considerably enriched in tubulin (as judged by SDS-PAGE) and containing most of the colchicine-binding activity originally present in

the synaptosol (see Lagnado et al. 1971); thus extending the evidence that a soluble form of tubulin, which might have originated from microtubules, was present in isolated nerve endings preparations (= synaptosomes).

The recent discovery that microtubules can be detected in nerve endings in situ, where they appear to be closely associated with both presynaptic and postsynaptic membrane elements (Gray, 1975; Westrum and Gray, 1976), indicates that part, at least, of the tubulin pool detected in isolated synaptosomes might be competent for reassembly in vitro. This possibility is considerably strengthened by recent observations that microtubules can also be seen in isolated synaptosomes (Gray, E.G. personal communication; Hajos, F. personal communication).

Therefore, based on the above information, the in vitro reassembly of microtubules from synaptosomal soluble extracts was investigated under assembly conditions identical to those used to polymerize tubulin from crude high-speed extracts of brain. In this work, electron microscopy (using negative staining techniques) was employed to monitor the formation of polymerized structures. Parallel experiments were also conducted to investigate microtubule assembly in the post-nuclear ( $S_1$ ) and post-mitochondrial ( $S_2$ ) soluble fractions derived from the same preparations (i.e., 10% (w/v) homogenates of chick brain), since virtually all previous studies on tubulin assembly were solely based on high-speed supernatants from concentrated (i.e., 40-50% (w/v) homogenates of brain. Microtubule assembly in concentrated extracts of 1-3 day-old chick brain was also examined (by electron microscopy) since, to my knowledge, all previous work showing microtubule assembly in vitro was based on the use of mammalian brain.

For the reasons discussed at the end of the previous chapter, a new procedure was introduced to fractionate homogenates of chick brain. It was thought useful, at this stage, to investigate the subcellular distribution of proteins and colchicine-binding activity in tissue fractionated by the method of Leeuwen et al. (see below) before proceeding to the polymerization experiments. During this work, the protein patterns of the isolated fractions was also examined by SDS-PAGE in order to further characterize them and to attempt to determine whether any obvious correlation could be established between the tubulin-comigrating protein and the distribution of colchicine-binding activity amongst the various purified subcellular fractions.

## 4.2    Methods

### 4.2.1    Subcellular fractionation

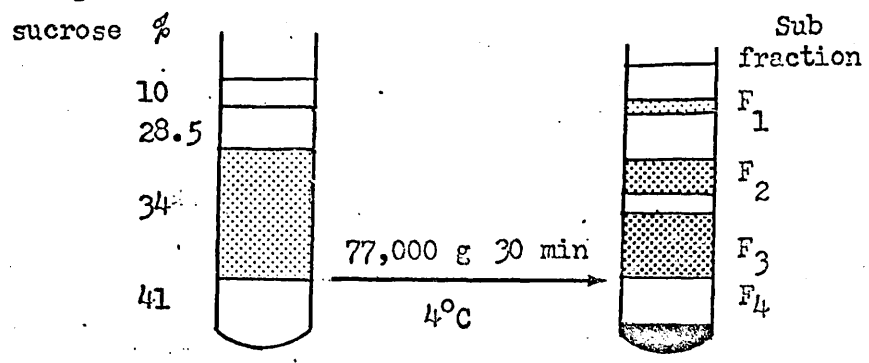
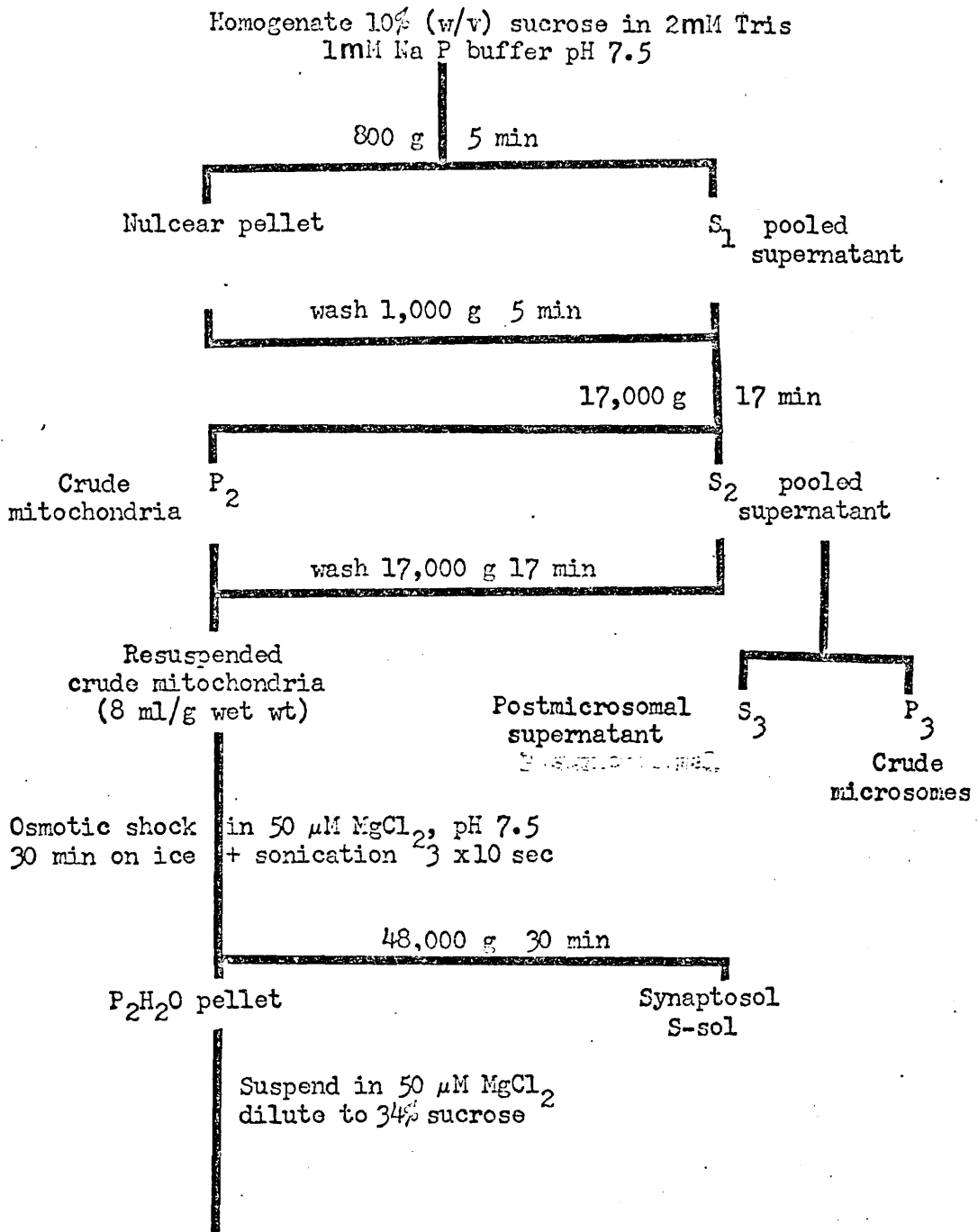
Since 1-3 day-old chicks were readily available at the time this particular work was carried out, the fractionation method followed to extract synaptosomes, was that described by Van Leeuwen et al. (1976) using chick brain tissue. This method was mainly based on the floatation-sedimentation centrifugation technique adapted from Jones and Matus (1974) and described in the previous chapter (see section 3.2.1).

The present fractionation procedure is diagrammatically illustrated in figure 4.1. A 10% homogenate from freshly excised whole chick brain was prepared in a medium consisting of 0.32 M sucrose, 2 mM Tris buffered with 1 mM phosphate pH 7.5. Following the first centrifugation (800 g for 5 min), the nuclear pellet was washed by resuspension in the same medium (to half the original volume of the homogenate) and centrifugation



Fig. 4.1 Diagrammatic scheme for the subcellular fractionation of chick brain by the method of Van Leeuwen et al.

Subfractions of the lysed crude mitochondria were separated on a sucrose gradient based on the floatation-sedimentation centrifugation technique of Jones and Matus (see text, section 4.2.1). Four subfractions were obtained: F<sub>1</sub>, myelin + 'light' membranes; F<sub>2</sub>, synaptic plasma membranes; F<sub>3</sub>, 'light' mitochondria + synaptic ghosts; F<sub>4</sub>, 'heavy' mitochondria. (see also text, section 4.2.1).





for 5 min at 1000 g. The first supernatant and wash were pooled to form the postnuclear supernatant ( $S_1$ ).  $S_1$  was centrifuged at 17,000 g for 17 min to give the crude mitochondrial fraction which was also washed with buffered sucrose (vol = half that of  $S_1$ ) and pelleted by spinning for 17 min at 17,000 g. Once more, the supernatant and wash were pooled to give the postmitochondrial supernatant or  $S_2$ . This latter was further centrifuged at 100,000 g for 60 min to obtain the microsomes  $P_3$  and post-microsomal supernatant  $S_3$ . The washed crude mitochondrial fraction was then osmotically lysed in 50  $\mu$ M  $MgCl_2$  (8 ml/g original wet wt of tissue) by gentle homogenization (6 strokes) and sonication in a 150 watt MSE ultrasonic desintegrator operated at medium power with optimal amplitude. The suspension was readjusted to pH 7.5 with NaOH and left on ice for 30 min. The pellet ( $P_2-H_2O$ ) obtained by spinning the sonicated preparation for 30 min at 48,000 g was resuspended in 50  $\mu$ M  $MgCl_2$ , brought up to 34% (w/v) with sucrose and overlaid on a 41% (w/v) sucrose layer (see fig. 4.1). This was carefully topped by a 28.5% sucrose phase and finally overlaid with 10% (w/v) sucrose. The whole gradient was centrifuged at 77,000 g for 30 min and, according to Leeuwen et al., the four fractions collected from the three interphases and the pellet can be described, on the basis of electron microscopy and enzymic profiles, as follows:-

- 1- Fraction 1, ( $F_1$ ) recovered at the 10% and 28.5% interphase and consisting mainly of myelin.
- 2- Fraction 2, ( $F_2$ ) recovered at the 28.5% and 34% interphase, and consists mainly of synaptic plasma membranes.
- 3- Fraction 3, ( $F_3$ ) collected at the 34% and 41% interphase contains a mixture of synaptic ghosts and "light" mitochondria.

4- Fraction 4, ( $F_4$ ) a brown pellet, which consists mostly of "heavy" mitochondria.

#### 4.2.2 Colchicine-binding activity

Samples from all the fractions and subfractions, obtained by this method, were incubated at 37°C for 90 min in the presence of  $^3\text{H}$  colchicine. The incubation mixture consisted of sodium phosphate buffer pH 6.8 with 400-500 $\mu\text{g}$  of sample proteins and 5  $\mu\text{M}$  final concentration of colchicine. The binding reaction was stopped on ice and each sample assayed, separately, for binding activity using the DE 81 filter disc method (see Methods chapter, section 2.3.2).

#### 4.2.3 Gel electrophoresis

Samples from the same above fractions were loaded on SDS polyacrylamide gels for proteins separation. Duplicates of these gels were run in the presence of purified tubulin as a marker (see Methods chapter, section 2.7).

#### 4.2.4 Polymerization

Samples from  $S_1$ ,  $S_2$  and the synaptosol, with enough proteins, were used for polymerization. This was, in the first instance, performed in the presence of glycerol, MES, EGTA, GTP and  $\text{MgCl}_2$  (Shelanski et al. 1973). The possibility of polymerization in the synaptosol fraction under different conditions, was also investigated. These were mainly:-

- a- In the presence of glycerol, MES, EGTA, GTP, EDTA.
- b- In the absence of glycerol (see Borisy et al. 1975).
- c- In the presence of relatively very small volumes (0.5-1 ml, depending on the volume of solution to be polymerized) of  $S_1$  and  $S_2$  in the

synaptosol using the Shelanski polymerization conditions. In this case, the control contained the same added volumes of  $S_1$  and  $S_2$  but with polymerization buffer to replace the synaptosol.

#### 4.2.5 Electron microscopy

Towards the end of the incubation, samples from the incubated solutions were mounted on 400 F grids. A drop of warm 1% glutaraldehyde (37°C) was added and washed twice with warm distilled water (37°C). For fixation, 1% uranyl acetate was used (see Methods chapter, section 2.10). The grids were finally viewed under electron microscope, and photographs with different magnifications from representative areas taken for each preparation (see Methods chapter, section 2.10).

#### 4.2.6 Protein determination

The contents and distribution of proteins in the different fractions obtained were determined using the Folin Lowry method (see Methods chapter, section 2.6.1).

### 4.3 Results

#### 4.3.1 Distribution of protein and colchicine-binding activity

The results summarised in table 4.1 show that the distribution of the binding activities and the enrichments in the primary fractions  $P_2$ ,  $S_2$ ,  $P_3$ , and  $S_3$  were similar to those obtained in the previous chapter using the Jones and Matus method (see section 3.3.2, and fig. 4.2 A & B, 4.3 A & B). However, the distribution of protein and colchicine-binding (CB) activity between the soluble (synaptosol) and particulate ( $P_2-H_2O$ ) differed markedly in the two sets of experiments. Using the present procedure, 39%

and 68% of the protein and CB activity, respectively, were recovered in the soluble fraction giving an RSA value of 1.74 for the synaptosol (see table 4.1 and fig. 4.3A). This is in marked contrast to the results found in Chapter III (table 3.1 and fig. 3.8), where only 23 and 33%, respectively, of the protein and CB activity were recovered in the S-sol fraction giving rise to a lower RSA value for this fraction of 1.43. In fact, if one compares the ratio of the percentage distribution of CB activity between the soluble and particulate fractions obtained by both methods, it is found to be four times as high under the present experimental conditions. These differences may be related to the observation that the crude mitochondrial preparation ( $P_2$ ), from which the soluble and particulate fractions were derived, accounted for about 27% of the CB activity present in the mother fraction  $S_1$  (see table 4.1) as compared to only 19% in the earlier experiments (see table 3.1).

Furthermore, when one compares the data for the particulate subfractions of  $P_2$  using both methods, additional differences were also observed in the distribution both of the protein and CB activities. Thus, it can be readily seen from the data summarised in tables 3.1 and 4.2 that there is a redistribution of both protein and CB activity amongst the two lighter fractions obtained by either methods. In particular, it is noted that the fraction enriched in SPM obtained by the Leeuwen method, ( $F_2$ ), is considerably enriched in CB activity as compared to that obtained by Jones and Matus. This seems to be due, in part at least, to the redistribution that occurred between the myelin and SPM fractions (i.e.,  $F_1$  and  $F_2$ ). This however did not result in a specific enrichment of CB activity in the myelin fraction, as can be seen by comparing the RSA values for this fraction (see fig. 3.8 and 4.3B). Presumably, therefore, light membranes poorer in CB activity

were floated into the myelin fraction by the Leeuwen procedure.

The data in table 4.2 also indicate that despite the additional resolution of the "mitochondrial fraction" into a fraction containing light mitochondria plus synaptic ghosts ( $F_3$ ) and one containing purified "heavy mitochondria" ( $F_4$ ), a substantial part of both CB activity and protein was recovered in the pellet fraction obtained by both procedures. Nonetheless, neither of these fractions was enriched in CB activity when this is expressed in term of RSA values. It is perhaps noteworthy that the  $F_3$  fraction showed the lowest RSA value of all the fractions examined by either methods (cf. fig. 3.6 & 4.3B). Whether in fact the substantial CB activity observed in the mitochondria fraction was due to the tubulin-like protein seems unlikely in view of the lack of stimulation of CB activity in the fraction by vinblastine (see table 3.1). However, further work is required to clarify the nature of the CB sites associated with the mitochondrial fraction and to establish whether this represents typical specific binding sites characteristic of tubulin.

Incidentally to the work described above, it was found that when the crude mitochondrial fraction was lysed by gentle homogenization as opposed to sonication, less protein could be recovered in the soluble (synaptosol) fraction as might be expected. On the other hand, it can be seen from the data summarised in table 4.3 that this was not paralleled by a similar solubilization of CB activity. From a comparison of the RSA values obtained it is evident that the gentler homogenization procedure resulted in a preferential release of CB protein. The reason for this difference does not appear to be related to a selective inactivation of CB activity by sonication, since this treatment in fact slightly increased the total activity recovered from the two fractions (i.e.,  $P_2-H_2O + S-sol$ ).

Table 4.1 Distribution of colchicine-binding activities in primary subfractions of chick brain homogenate using the Van Leeuwen et al. method.

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Results shown are means of triplicate determinations from four separate experiments (<10% difference between experiments). Values in the first two columns are given as a percentage of protein or binding activity recovered from mother fractions: homogenate; S<sub>1</sub>; S<sub>2</sub>; P<sub>2</sub>. RSA, relative specific activity, = % bound c.p.m. recovered/% protein recovered. Binding activities were assayed radiometrically as described in Chapter II, section 2.3.2.(see also details in text, section 4.3.1).



Primary fraction	Sub fraction	Description	Protein distribution %	Colchicine distribution %	RSA
Homogenate	N	Nuclear pellet	7.0	8.5	1.21
	S <sub>1</sub>	Post nuclear supernatant	93.0	91.5	0.98
S <sub>1</sub>	P <sub>2</sub>	Mitochondrial pellet	33.0	26.5	0.80
	S <sub>2</sub>	Post mitochondrial supernatant	67.0	73.5	1.10
S <sub>2</sub>	P <sub>3</sub>	Microsomal pellet	56.0	41.5	0.74
	S <sub>3</sub>	Post microsomal supernatant	44.0	58.5	1.33
P <sub>2</sub>	P <sub>2</sub> H <sub>2</sub> O	Lysed mitochondria particulate	61.0	32.0	0.53
	S-sol	Lysed mitochondrial supernatant	39.0	68.0	1.74
		= synaptosol			

Table 4.1



The first part of the document is a list of names and titles, including "The Hon. Mr. Justice" and "The Hon. Mr. Justice". The text is somewhat faded and difficult to read, but appears to be a list of individuals involved in a legal or official proceeding.

The second part of the document contains several paragraphs of text, which are also somewhat faded. The text appears to be a formal report or a set of proceedings, possibly related to the individuals listed in the first part. The content is largely illegible due to the quality of the scan.

Fig. 4.2 Relative distribution of the colchicine-binding activity in primary subcellular fractions of chick brain (see text, section 4.3.1).

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Subcellular fractionation was carried out using the method of Van Leeuwen et al. to yield fractions: S<sub>2</sub>, postmitochondrial supernatant; P<sub>2</sub>, lysed crude mitochondria; S<sub>3</sub>, postmicrosomal supernatant; P<sub>3</sub>, crude microsomes. RSA = % bound c.p.m. recovered/% protein recovered.

Fig. 4.3 Relative distribution of the colchicine-binding activity in subfractions of lysed crude mitochondria from chick brain (see text, section 4.3.1).

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Lysed crude mitochondria were first separated into a mitochondrial particulate (P<sub>2</sub>H<sub>2</sub>O) and mitochondrial soluble extract (S-sol). P<sub>2</sub>H<sub>2</sub>O was subfractionated, by the floatation-sedimentation centrifugation technique, into four fractions; F<sub>1</sub>, myelin + "light" membranes; F<sub>2</sub>, synaptic plasma membranes; F<sub>3</sub>, "light" mitochondria + synaptic ghosts; F<sub>4</sub>, "heavy" mitochondria. RSA = % bound c.p.m. recovered/% protein recovered.

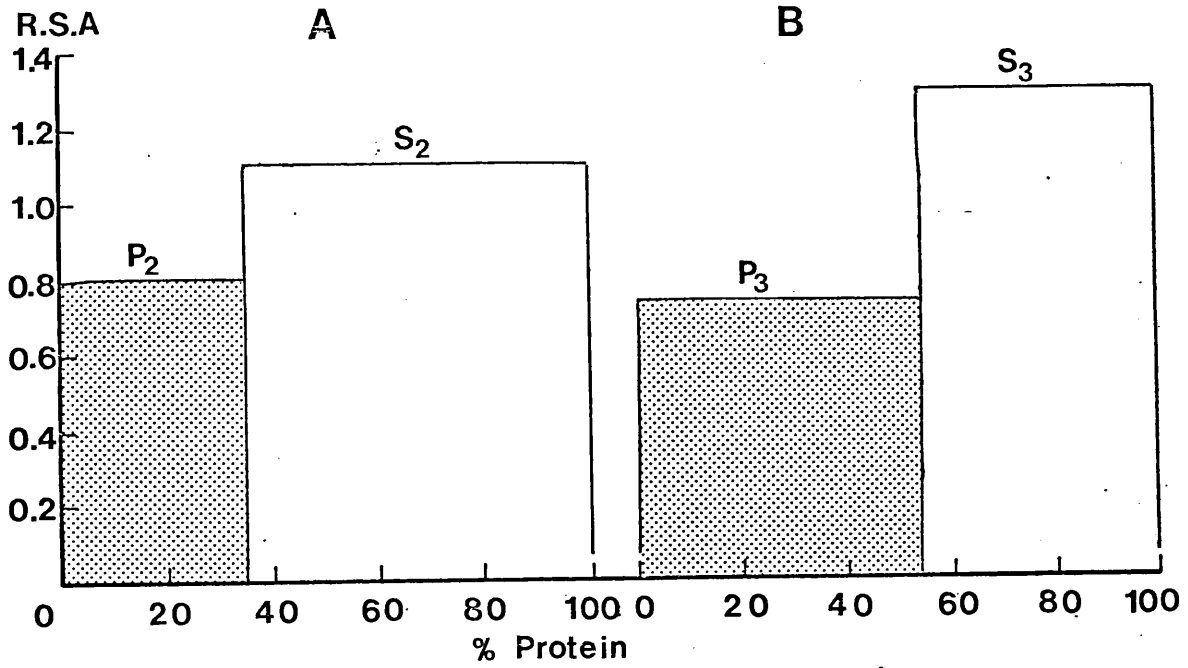


Fig.4.2

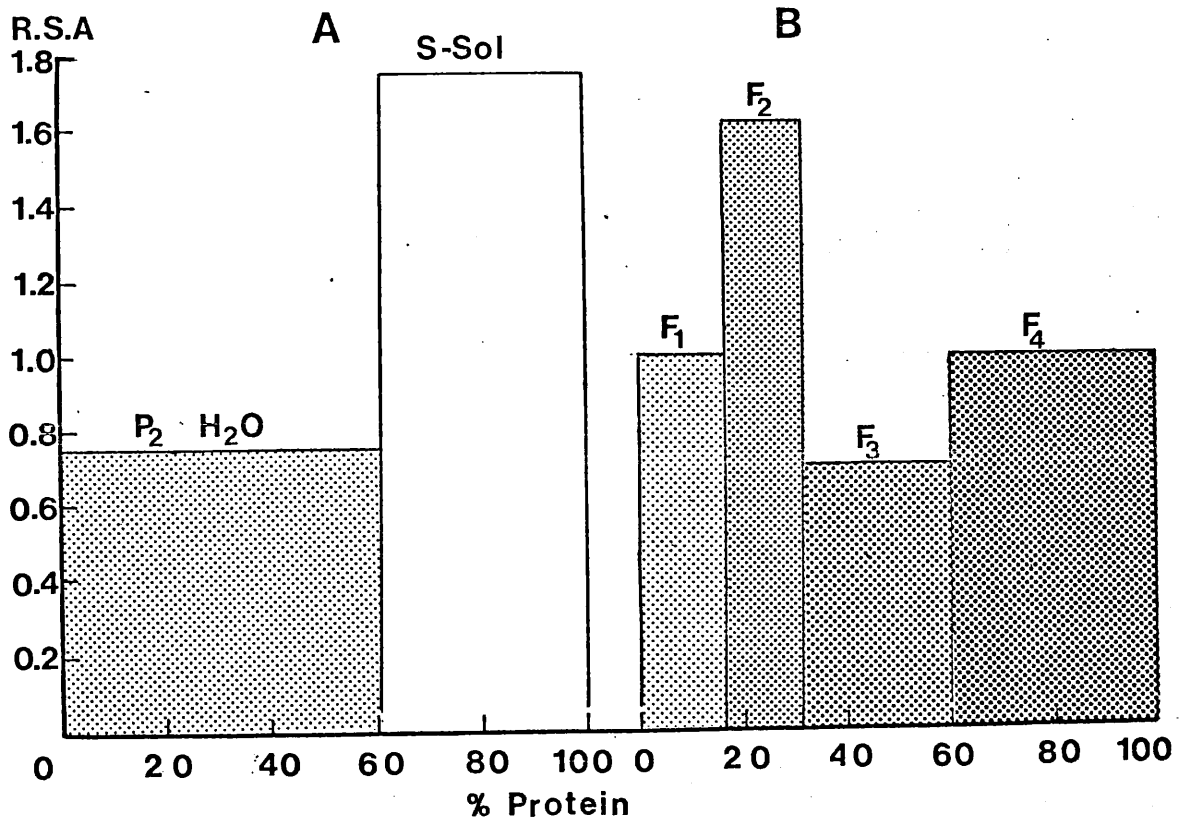


Fig.4.3



Table 4.2 Distribution of colchicine-binding activities in subfractions of lysed crude mitochondria from chick brain: a comparison between the Van Leeuwen et al. and Jones & Matus methods.

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Results are means of triplicate determinations from three separate experiments ( $< 10\%$  difference between experiments). Values in the first two columns, in each method, are given as a percentage of protein or binding activity recovered from the lysed mitochondrial particulate ( $P_2H_2O$  or  $P_{O_2}$  respectively). RSA, relative specific activity, =  $\%$  bound c.p.m. recovered/ $\%$  protein recovered. Binding activities were assayed radiometrically as described in Chapter II, section 2.3.2 (see also details in text, section 4.3.1).

P<sub>2</sub>H<sub>2</sub>O subfractions by Leeuwan method

P<sub>2</sub>H<sub>2</sub>O subfractions by Jones and Matus method

Protein Colchicine  
distribution distribution  
% %  
R.S.A R.S.A

Sub fraction Description Description

F <sub>1</sub>	Myelin + "light" membranes	16.1	16.0	0.99	Myelin (My)	6	6	1.00
F <sub>2</sub>	Synaptic plasma membranes	15.7	25.5	1.63	Synaptic plasma membranes (SPM)	25	34	1.36
F <sub>3</sub>	"Light" mitochondria + synaptic ghosts	27.5	18.0	0.66	Mitochondria (Mit)	69	60	0.87
F <sub>4</sub>	"Heavy" mitochondria	40.7	41.5	1.00				

Table 4.2

Treatment of P <sub>2</sub> fraction	Sub fraction	Total protein per fraction mg	Protein distribution %	Total cpm per fraction x 10 <sup>6</sup>	cpm distribution %	PSA
Sonication	P <sub>2</sub> H <sub>2</sub> O	177	61.5	1.33	30.5	0.50
	S-sol	110	38.5	3.07	69.5	1.81
Homogenization	P <sub>2</sub> H <sub>2</sub> O	221	76.2	1.52	38.5	0.51
	S-sol	69	23.8	2.44	61.5	2.58

**Table 4.3** Distribution of colchicine-binding activity in the lysed mitochondrial particulate (P<sub>2</sub>H<sub>2</sub>O) and synaptosol (S-sol) fractions prepared according to the Van Leeuwen *et al.* method.

Sonication was performed using a 150 watt MSE ultrasonic desintegrator; homogenization was by 6 gentle strokes in a glass-teflon homogenizer (see also text, section 4.3.1).

## 4.3.2 Gel electrophoresis

### 4.3.2.1 Primary soluble fractions

Samples from  $S_1$  and  $S_2$  were run on SDS-PAGE in the presence of urea. The pattern of proteins obtained in each case, showed clearly the presence of a double band corresponding to  $\alpha$  and  $\beta$  tubulin (MW 54,000 and 57,000) on the basis of its comigration with purified tubulin added as a marker (see fig. 4.4). This was confirmed by gel scans (data not shown) which indicated that tubulin represents one of the major proteins present in these extracts.

### 4.3.2.2 Mitochondrial particulate subfractions

Amongst these fractions,  $F_2$  and  $F_3$  showed the presence of  $\alpha$  and  $\beta$  tubulin comigrating protein, as well as an actin-like protein (ALP) band (MW 47,000; see fig. 4.5 A & 4.5 B). Surprisingly,  $F_1$  also showed, very clearly, the presence of a double band comigrating with tubulin (see fig. 4.6) which is in keeping with the presence of significant colchicine-binding activity observed in this fraction (see section 4.3.1 and table 4.2). Recentrifugation of the  $F_1$  fraction over a 9% sucrose solution removed some, but not all of the tubulin material present in this fraction (fig. 4.6).

### 4.3.2.3 The synaptosol

The nature of proteins present in this fraction was investigated by two steps:-

- First, samples from the whole synaptosol were loaded on gels. In the region where a double band of tubulin was expected, only one single major band



apparently comigrated with purified  $\alpha$ -tubulin (see fig. 4.7 A). However, part of this  $\alpha$ -like band seemed to migrate slightly more slowly than the  $\alpha$  tubulin component of the marker. Later, when the load of the gels with synaptosomal proteins was reduced, the  $\alpha$ -like band was found to consist of a tightly arranged doublet, which is referred to as  $\alpha_1$  and  $\alpha_2$  in figure 4.7 A, where the faster ( $\alpha_2$ ) component is seen to comigrate with  $\alpha$ -tubulin. High molecular weight proteins (HMW): two bands of MW in the region of 330,000, and intermediate molecular weight bands (IMW): two bands, MW ca. 80,000 and 100,000, were also observed on the same gels, together with a fast travelling band, ahead of tubulin (MW ca. 47,000) that is referred to as ALP (see fig. 4.7 A).

— Second, a sample from the same synaptosomal was incubated with vinblastine ( $5 \times 10^{-4}$  M) for 15 min at  $37^\circ\text{C}$  with a view to selectively precipitating tubulin (see Methods chapter, section 2.2.2). The centrifuged precipitate was resuspended in sample buffer and run on gels. In this case, a double band comigrating with the  $\alpha$  and  $\beta$  polypeptides of marker tubulin was clearly observed (see fig. 4.7 B), as well as a band of MW ca. 47,000, referred to earlier as ALP. The  $\alpha$ -like band of the vinblastine precipitate seemed to correspond to the faster  $\alpha_2$  component of tubulin-like protein seen in unfractionated synaptosomal (see fig. 4.7 B and above "first step"). Both of the HMW bands were also precipitated by vinblastine, while only a slight amount of the 80,000 band seemed to be brought down.

The data obtained using either added marker tubulin, or vinblastine, both point to the existence of relatively low concentrations of tubulin in the original synaptosomal extracts where the main band seen in the vicinity of tubulin occupies a region which does not strictly coincide with either  $\alpha$  or  $\beta$  tubulin. It is possible that this main band contains

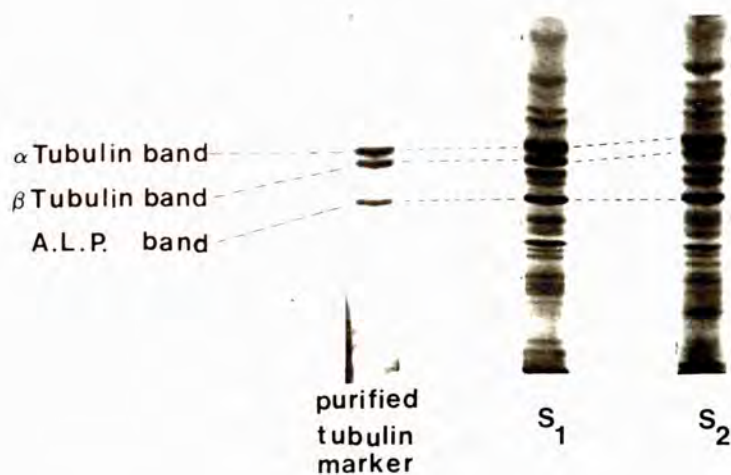


Fig. 4.4 Gel electrophoresis of primary soluble fractions.

Samples from S<sub>1</sub>, postnuclear, and S<sub>2</sub>, postmitochondrial, fractions were run on SDS-PAGE in the presence of urea. Electrophoresis was carried out in tris-glycine buffer system. S<sub>1</sub> and S<sub>2</sub> gels were compared against a marker of purified tubulin showing clearly the  $\alpha$  and  $\beta$  tubulin (ca. 54,000 and 57,000 MW, respectively), as well as the actin-like protein band (A.L.P., 47,000 MW). See also text, 4.3.2.1.



Fig. 4.5 A Gel electrophoresis of lysed mitochondria subfractions.

Samples from F<sub>2</sub> (synaptic plasma membranes fraction) were run on SDS-PAGE in the presence of urea. Electrophoresis was carried out in tris-glycine buffer system. The gel shows clearly the presence, in F<sub>2</sub>, of a double band comigrating with the  $\alpha$  and  $\beta$  tubulin of the marker (54,000 and 57,000 MW, respectively). A band comigrating with the actin-like protein (ALP) of the marker was also observed (see text, section 4.3.2.2).

Fig 4.5 B Gel electrophoresis of lysed mitochondria subfractions.

Samples from F<sub>3</sub> ("light" mitochondria + synaptic ghosts fraction) were run on SDS-PAGE as described above. Bands comigrating with  $\alpha$  and  $\beta$  tubulin, as well as the actin-like protein (ALP) were again observed in this fraction (see text, section 4.3.2.2).

F<sub>2</sub> = Synaptic Plasma Membranes

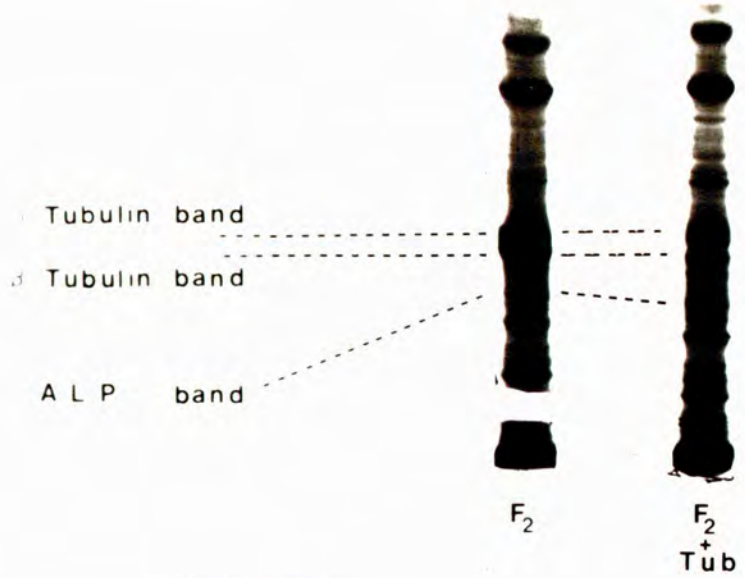


Fig. 4.5 A

F<sub>3</sub> = Light Mitochondria + Synaptic Ghosts

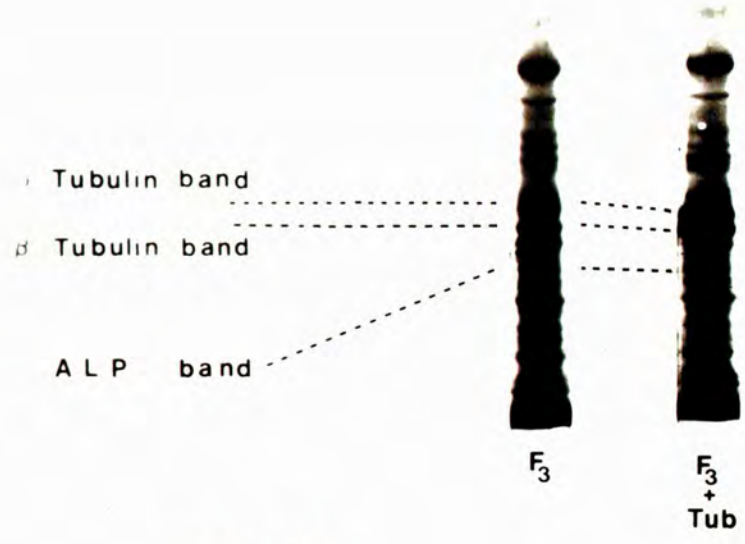


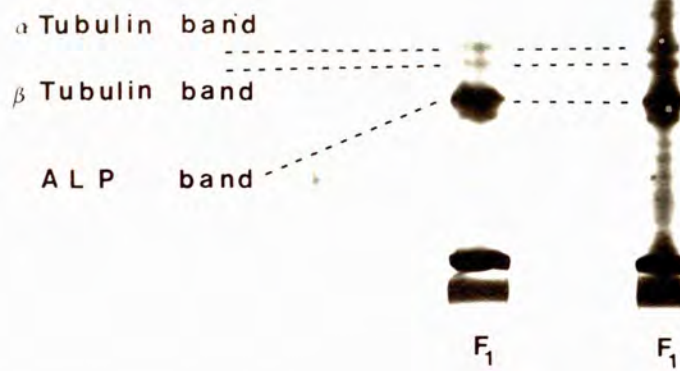
Fig. 4.5 B



Fig. 4.6 Gel electrophoresis of lysed mitochondria subfractions.

Samples from F<sub>1</sub> (myelin + "light" membrane fraction), were run on SDS-PAGE in the presence of urea. Electrophoresis was carried out in tris-glycine buffer system. The gel showed very clearly the presence of a double band comigrating with tubulin. F<sub>1</sub> was also highly enriched in actin-like protein (ALP) as judged by the dye intensity. Further centrifugation of F<sub>1</sub> over a 9% sucrose solution removed some, but not all of the tubulin material present in this fraction (see upper gels; and text, section 4.3.2.2).

Purified Myelin fraction



Crude Myelin fraction

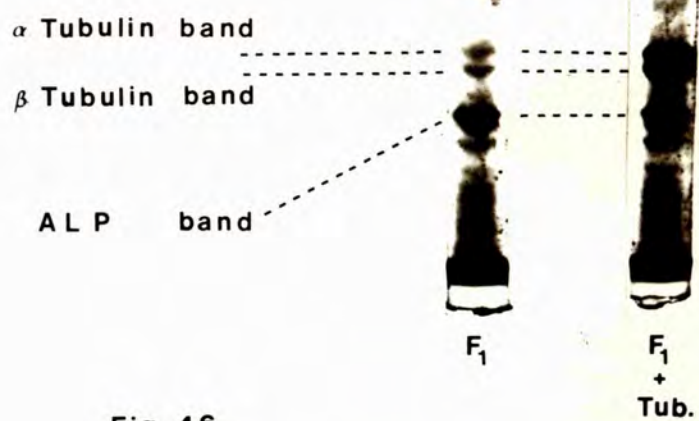


Fig. 4.6





Fig. 4.7 A Gel electrophoresis of synaptosol from lysed mitochondria.

Samples from S-sol, crude mitochondria supernatant or synaptosol, fraction were run on SDS-PAGE in the presence of urea. Electrophoresis was carried out in tris-glycine buffer system. A major single band comigrating with purified  $\alpha$ -tubulin was observed. It was found to consist of a tightly arranged doublet  $\alpha_1$  and  $\alpha_2$  where  $\alpha_2$  the faster component comigrated with purified  $\alpha$  tubulin. High molecular weight, (HMW), and intermediate molecular weight, (IMW), proteins were also observed, together with a fast travelling band, ahead of tubulin (MW ca. 47,000), referred to as ALP. HMW = 300,000 - 350,000 MW.; IMW = 80,000 & 110,000 MW.(see text, section 4.3.2.3).

Fig. 4.7 B Gel electrophoresis of vinblastine, (Vb), precipitate from synaptosol.

Samples from synaptosolic Vb-precipitate were run on SDS-PAGE as described above. A double band comigrating with the  $\alpha$  and  $\beta$  polypeptides of marker tubulin can clearly be observed, as well as a band of MW ca. 47,000, (ALP). The  $\alpha$ -like band of Vb precipitate seems to correspond to  $\alpha_2$  component of tubulin-like protein seen in untreated synaptosol. Both of the HMW bands were precipitated by Vb, as well as slight amount of the 80,000 MW band. See also text, section 4.3.2.3.

Fig. 4.7 A

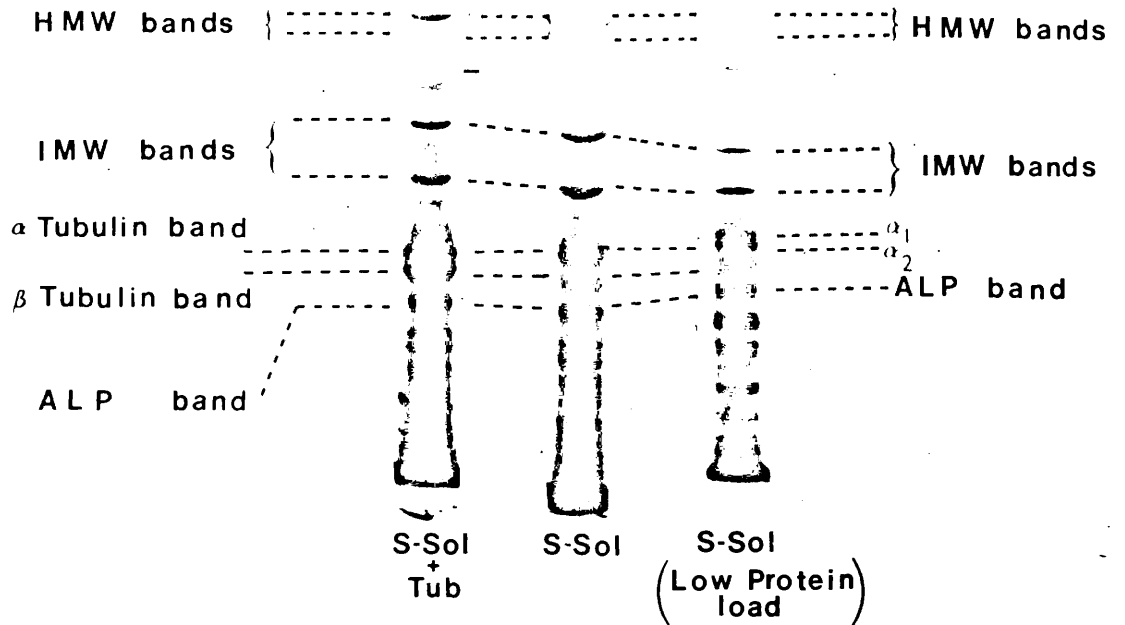
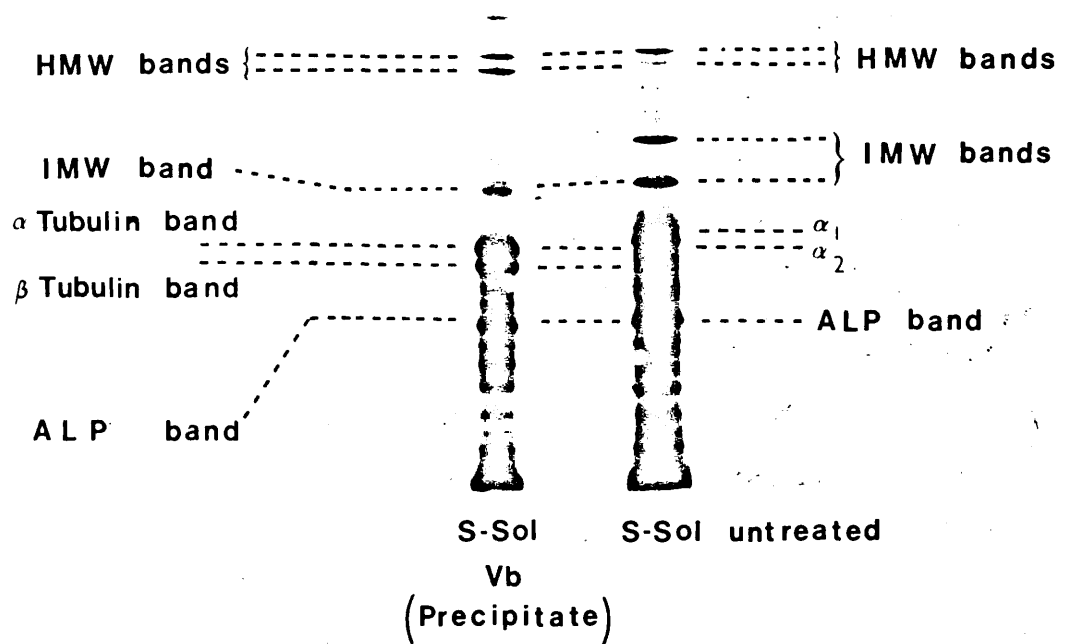


Fig. 4.7 B



both  $\alpha$  and a non-tubulin comigrating protein since one would expect  $\alpha$  and  $\beta$  subunits to be roughly in equal proportion. The observation that vinblastine precipitates are apparently enriched in both the  $\alpha$  and  $\beta$  components indicates, nevertheless, that they are both present in the synaptosol. An alternative interpretation would be that the synaptosol, as compared to whole brain extracts, contains mainly  $\alpha$  form of tubulin, and on this basis one might expect different kinds of microtubular polymers in nerve endings.

Clearly, the present semi-quantitative data, based on an electrophoretic system of limited resolution, do not allow to distinguish between these two possibilities. Thus, while the synaptosol fraction appears to be rich in colchicine-binding activity, it should be stressed that the total colchicine binding recovered in this fraction represents at most 12% of the total soluble CB activity present in chick brain homogenates (see table 4.1). This would be consistent with gel data showing that tubulin as represented by both  $\alpha$  and  $\beta$  subunits constitutes a relatively minor component of the synaptosolic protein on an absolute basis. This may explain the difficulties encountered in the attempts to demonstrate the assembly in synaptosol extracts as described below.

#### 4.3.3 Polymerization experiments

The assembly of any polymerisable protein present in the synaptosol was investigated mainly by electron microscopy viewing of the incubated preparation (see section 4.2.4, 4.2.5 and Methods chapter, section 2.10).

In these primary experiments, samples of the synaptosol,  $S_1$  and  $S_2$  soluble fractions were incubated in microtubule assembly buffer containing 4 M glycerol, following the procedure described by Shelanski et al. (1973)

(see Methods chapter, section 2.2.1 for details).

The micrographs obtained for the synaptosol showed no microtubules or any other filamentous structures (see fig. 4.11 A & 4.11 B). In incubated samples of  $S_1$  and  $S_2$ , however, microtubular structures of various lengths (up to several micrometers for  $S_1$  &  $S_2$ ) were observed (see fig. 4.9 A & B and 4.10 A & B). In the case of  $S_1$ , micrographs also showed ring structures (ca. 20 nm in diameter) in the background (see fig. 4.9 A & B). On the other hand, typical microtubules could be readily seen after incubation of high-speed extracts, from concentrated chick brain homogenates, during two polymerization cycles (see fig. 4.8), together with some ring structures.

#### 4.3.3.1 The effect of glycerol

In view of the reported polymerization of microtubules in the absence of glycerol (Borisy *et al.* 1975) attempts were made, in the present case, to investigate whether such condition could be favorable for successful assembly in the synaptosol, using  $S_1$  and  $S_2$  as controls. Both the synaptosol and  $S_2$  failed to give any sort of filamentous or ring structures (see fig. 4.13 A and 4.13 B). The only tubules obtained under this condition were from  $S_1$  (see fig. 4.12 A). These were 25 nm in width and their presence in any area of the grid was not as extensive as in the presence of glycerol (cf. fig. 4.12 B and 4.12 C).

#### 4.3.3.2 The effect of EDTA and Mg

The presence of Mg (0.5 mM) and other ions at very low concentrations had been shown to be essential for normal polymerization (see Introduction). However, these ions, at moderate to high concentrations ( $> 10$  mM for  $Mg^{++}$ , 1 mM for  $Ca^{++}$ , and 0.15 M for  $Na^+$  and  $K^+$ ) proved to be inhibitory for the

assembly process (see Olmsted and Borisy, 1975, and Borisy et al., 1975). Polymerization was therefore carried out on  $S_1$ ,  $S_2$  and the synaptosol following brain homogenization and synaptosome lysis in solution containing both EGTA and EDTA but no added  $MgCl_2$ . Incubation of samples in the glycerol-containing microtubule assembly buffer of Shelanski et al. (see section 4.3.3) resulted in the formation of relatively few, short microtubules from  $S_1$  and  $S_2$  (cf. fig. 4.14 A, B and 4.14 C for  $S_1$  and 4.15 A and 4.15 B for  $S_2$ ), while only ring structures (20 nm in diameter), similar to those previously seen in  $S_1$ , were observed after incubation of the synaptosol fraction under these conditions (see fig. 4.16 A & B).

#### 4.3.3.3 The effect of $S_1$ or $S_2$ presence

Since  $S_1$  and  $S_2$  were active sources of polymerization-competent tubulin (see above) and presumably contained tubulin associated proteins required for assembly, it was thought that their inclusion in the synaptosome might provide the required nucleating elements for the assembly of synaptosol tubulin, assuming that the concentration of this protein was sufficient. Therefore, assembly of synaptosol tubulin was investigated in material that was "spiked" with small volumes of  $S_1$  or  $S_2$ . In these experiments, controls were run using buffer (instead of synaptosol) containing similar amounts of  $S_1$  or  $S_2$ . The micrographs for "S-sol +  $S_1$ " (fig. 4.17 A & B) and "S-sol +  $S_2$ " (fig. 4.18 A & B) incubated in the presence of glycerol showed very few microtubules as compared to undiluted samples of  $S_1$  or  $S_2$  alone (see fig. 4.9 A,B & 4.10 A,B, respectively). Similarly, few microtubules were formed, however, when the diluted samples of  $S_1$  and  $S_2$  were incubated in the absence of synaptosol for control purposes (figs. 4.19 A,B and 4.20 A,B, respectively). This indicates that the microtubules formed in the combined fractions were primarily due to non-synaptosomal tubulin assembly. One possibility for

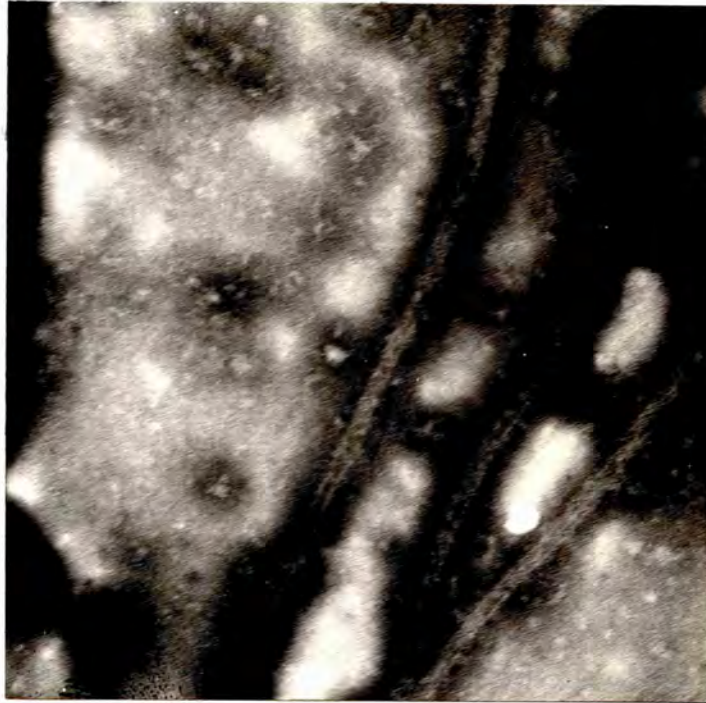


Fig. 4.8 Representative microtubules seen in samples of incubated high-speed extracts, from concentrated chick brain homogenates, during two polymerization cycles. Note also the presence of some ring structures. See text, section 4.3.3. Magnification = 84,000.





Fig. 4.9 A Representative single microtubule structure from postnuclear supernatant, S<sub>1</sub>, fraction. For details see below. Magnification = 190,000.

Fig. 4.9 B Representative microtubular structures (several micrometers in length), seen in samples of postnuclear supernatant (S<sub>1</sub>) incubated in microtubule assembly buffer in the presence of 4 M glycerol. Note the presence of several ring-like structures (15 - 25 nm in diameter). See text, section 4.3.3. Magnification = 87,000.

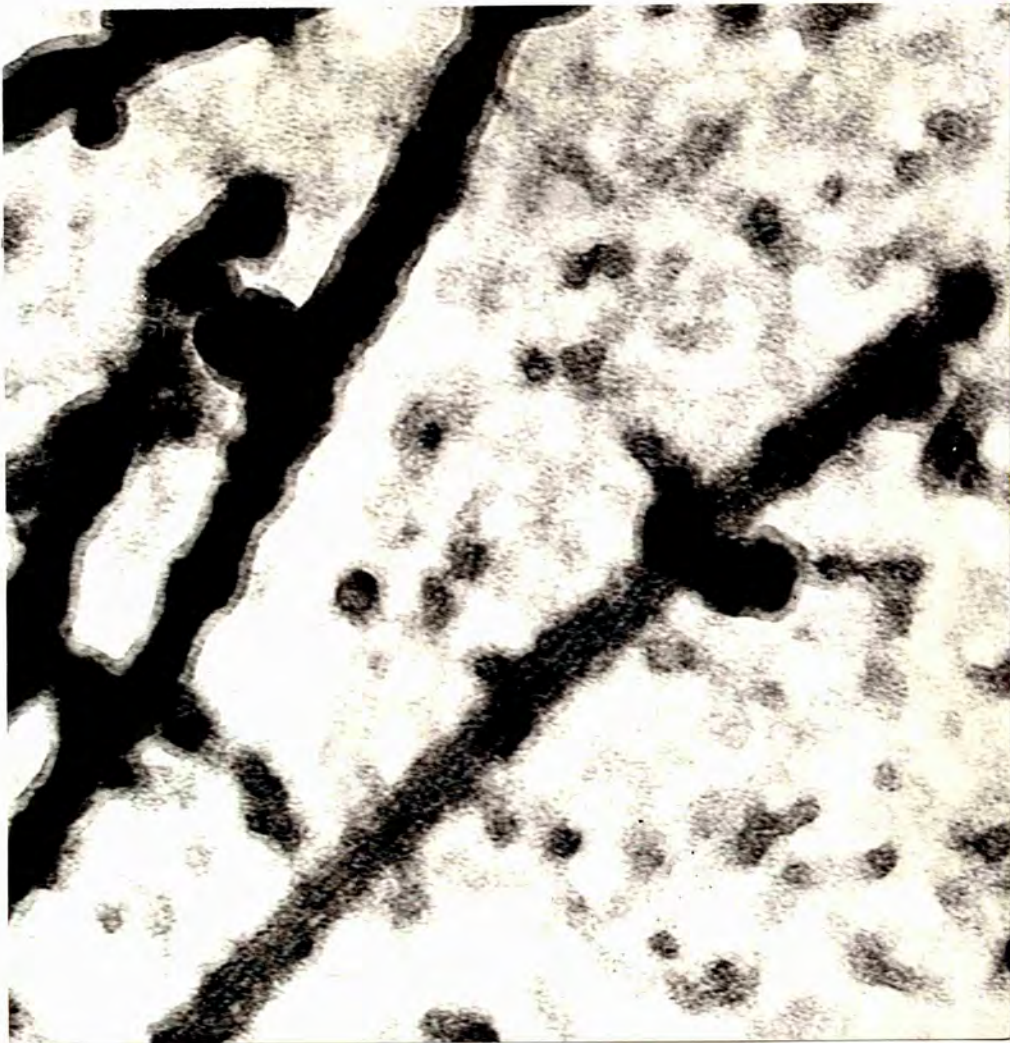


Fig. 4.9 A

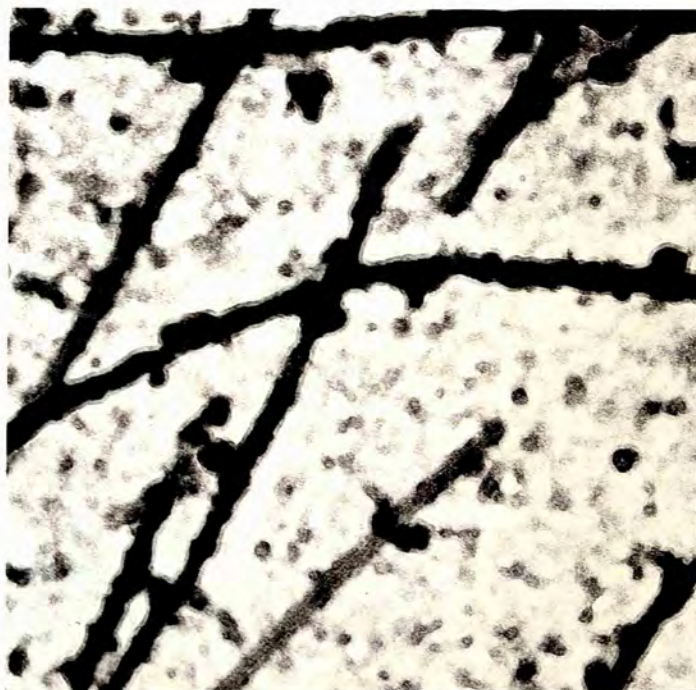


Fig. 4.9 B



Fig. 4.10 A Highly magnified microtubular structures from the postmitochondrial supernatant fraction, S<sub>2</sub>. For details see below. Magnification = 80,000.

Fig. 10 B Representative microtubular structures (several micrometers in length), seen in negatively stained samples of the postmitochondrial supernatant. Incubation was carried out in assembly buffer containing 4 M glycerol. The widths of the present structures are similar to those obtained from the postnuclear supernatant, S<sub>1</sub>, under the same conditions. Note the relative absence of ring structures. See also text, section 4.3.3. Magnification = 36,000.



Fig 4.10A



Fig.4.10B



Fig. 4.11 A Random field of lysed mitochondrial supernatant, or synaptosol, incubated in assembly buffer containing 4 M glycerol. No microtubules or any filamentous structures are observed (see text, section 4.3.3). Magnification = 7,500.

Fig. 4.11 B Random field of synaptosol fraction. Samples were incubated as described above (see also text, section 4.3.3). Magnification = 7,500.





**Fig. 4.11 A**



**Fig. 4.11 B**





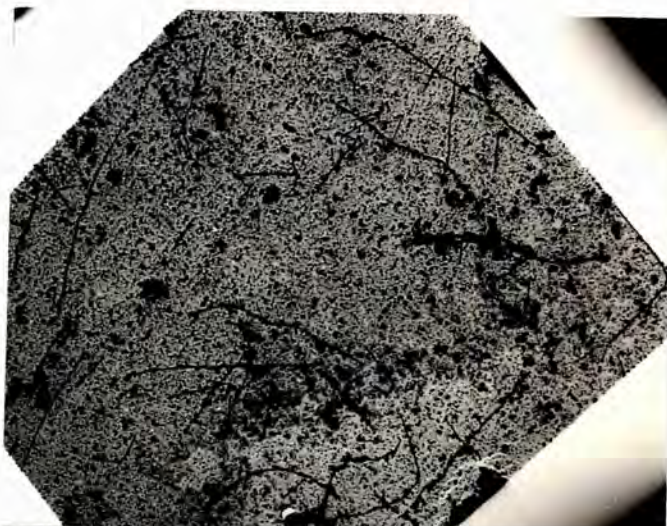
Fig. 4.12 A Representative tubular structures from samples of the postnuclear,  $S_1$ , fraction incubated in assembly buffer in the absence of glycerol. The observed width is about 25 nm. Note the presence of several ring-like structures. See also text, section 4.3.3.1. Magnification = 44,000.

Fig. 4.12 B Random field of the  $S_1$  fraction, incubated in assembly buffer in the absence of glycerol, showing the presence of very few microtubular structures formed under these conditions (compare with fig. 4.12 C below). See text, section 4.3.3.1. Magnification = 3,000.

Fig. 4.12 C Random field of the  $S_1$  fraction, incubated in assembly buffer in the presence of glycerol, showing the extensive presence of microtubular structures formed under such conditions (compare with fig. 4.12 B above). See also text, section 4.3.3.1. Magnification = 7,500.



Fi 4.12 A



Fi 4.12 B



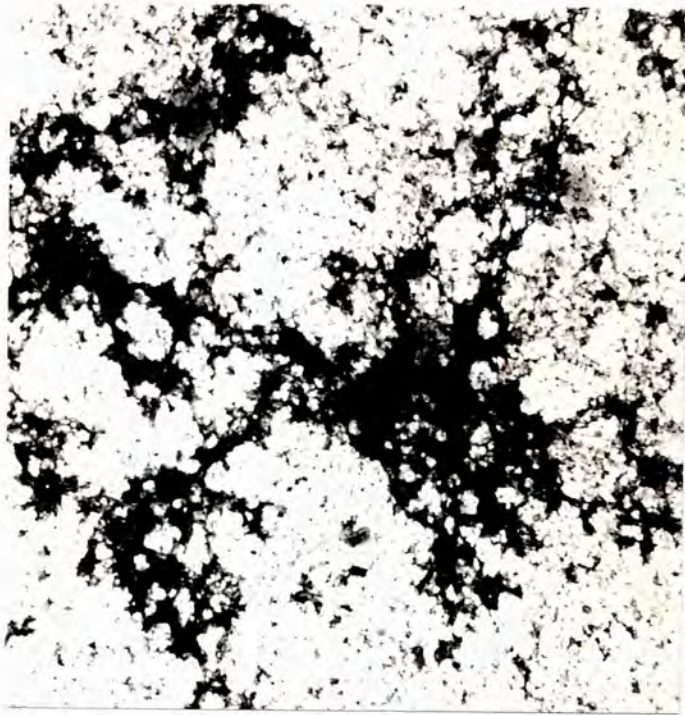
Fi 4.12 C



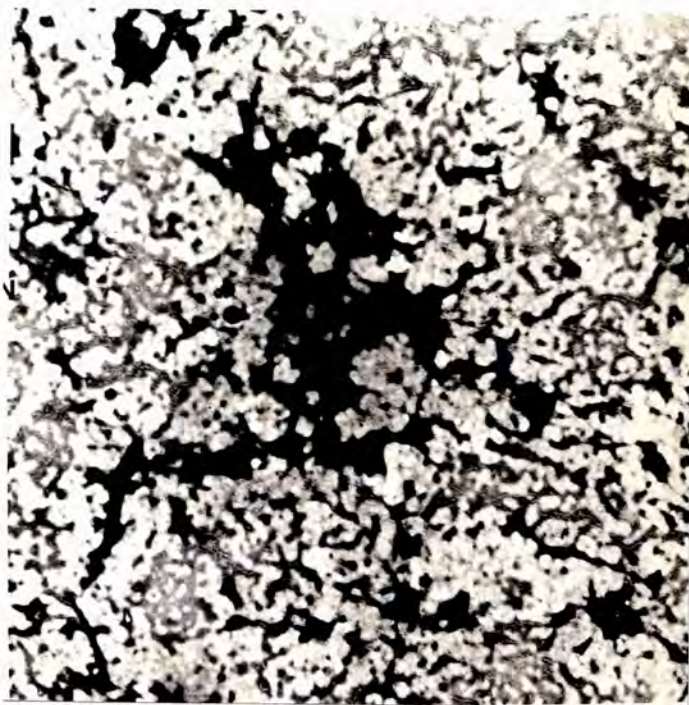
Fig. 4.13 A Micrograph resulting from the incubation of the lysed mitochondria supernatant, or synaptosol, in assembly buffer in the absence of glycerol. No filamentous or ring structures could be observed under such conditions (see text, section, 4.3.3.1). Magnification = 18,000.

Fig. 4.13 B Micrograph of samples from the postmitochondrial supernatant, S<sub>2</sub>, fraction incubated in assembly buffer in the absence of glycerol. No filamentous or ring structures could be observed under such conditions (see text, section 4.3.3.1). Magnification = 18,000.





**Fig. 4.13 A**



**Fig. 4.13 B**



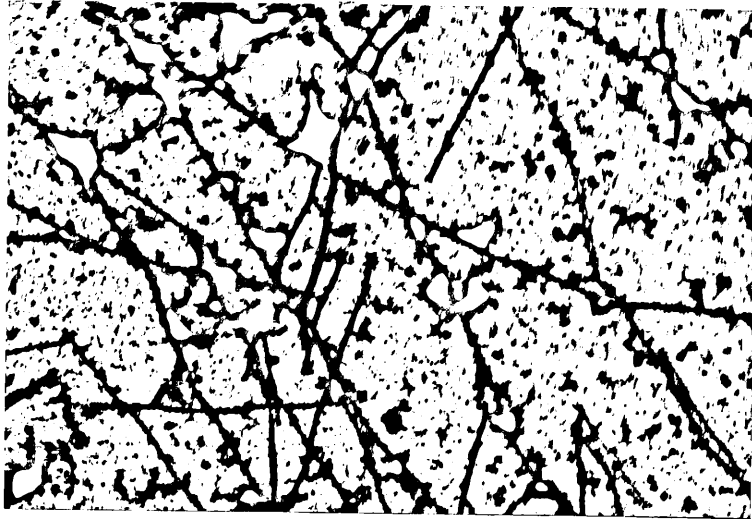
Fig. 4.14 A Microtubular structures from samples of  $S_1$  fraction extracted in the presence of EGTA and  $MgCl_2$ , and incubated in assembly buffer in the presence of glycerol. See text, section 4.3.3.2. Magnification = 22,000.

Fig 4.14 B Representative single microtubule obtained from samples of  $S_1$  fraction extracted and treated as described above. Magnification = 44,000.

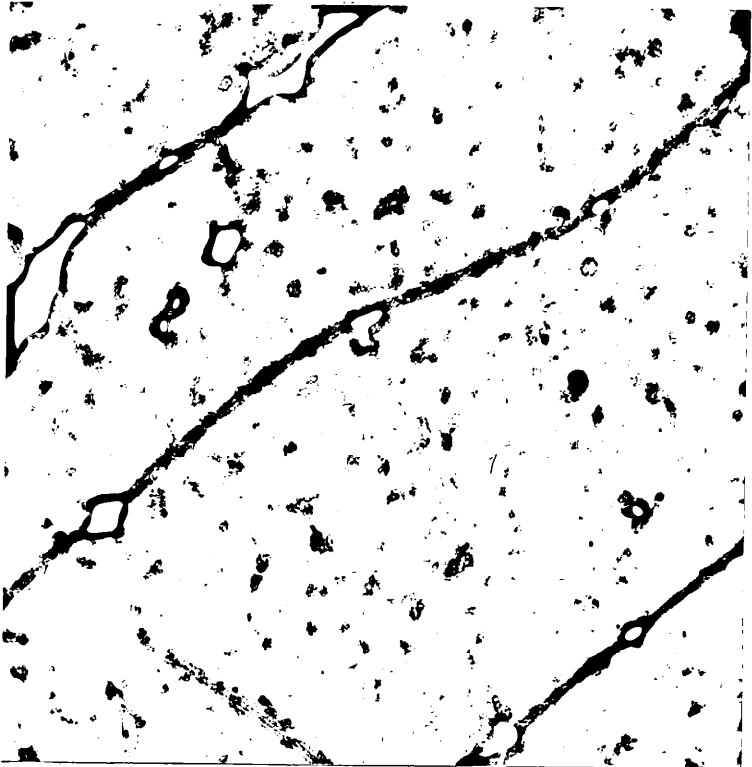
Fig. 4.14 C Microtubular structures from samples of  $S_1$  fraction extracted in the presence of EGTA, EDTA and without added  $MgCl_2$ . Incubation was carried out in microtubule assembly buffer in the presence of glycerol. Compared with fig. 4.14 A, relatively few and shorter microtubular structures are observed (see also text, section 4.3.3.2). Magnification = 22,000.



Fi 4.14 A



Fi 4.14 B



Fi 4.14 C

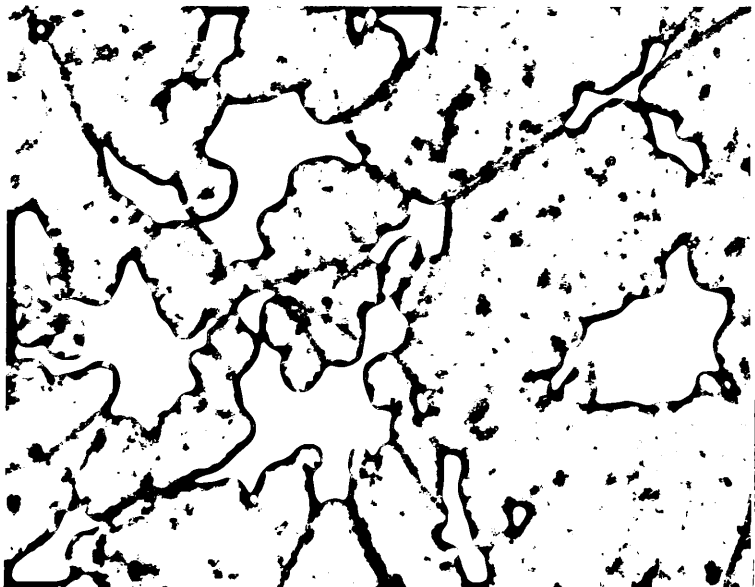




Fig. 4.15 A Microtubular structures from samples of S<sub>2</sub> fraction extracted in the presence of EGTA and MgCl<sub>2</sub>, and incubated in assembly buffer in the presence of glycerol. Microtubules are several micrometers in length. See also text, section 4.3.3.2. Magnification = 36,000.

Fig. 4.15 B Microtubular structures from samples of S<sub>2</sub> fraction extracted in the presence of EGTA, EDTA and without added MgCl<sub>2</sub>. Incubation was carried out in microtubule assembly buffer in the presence of glycerol. Compared with fig. 4.15 A, relatively fewer and shorter microtubular structures were observed (see also text, section 4.3.3.2). Magnification = 22,000.

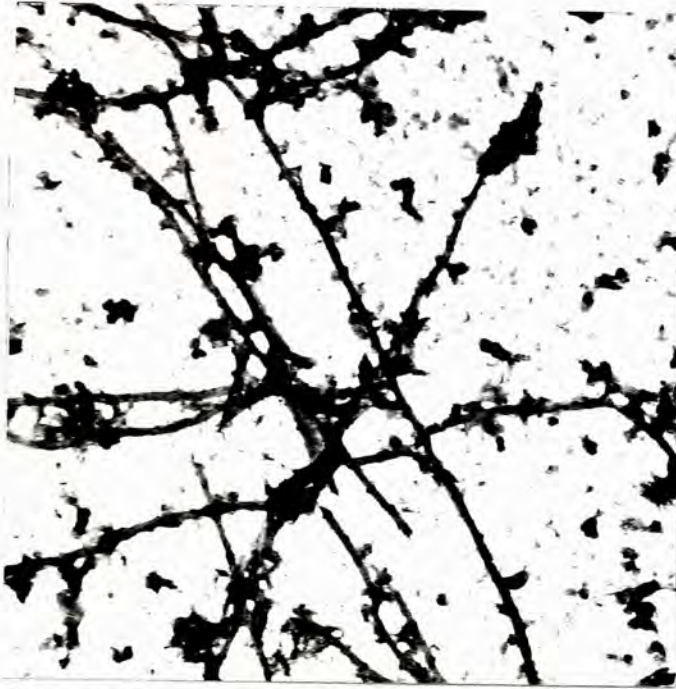


Fig. 4.15 A

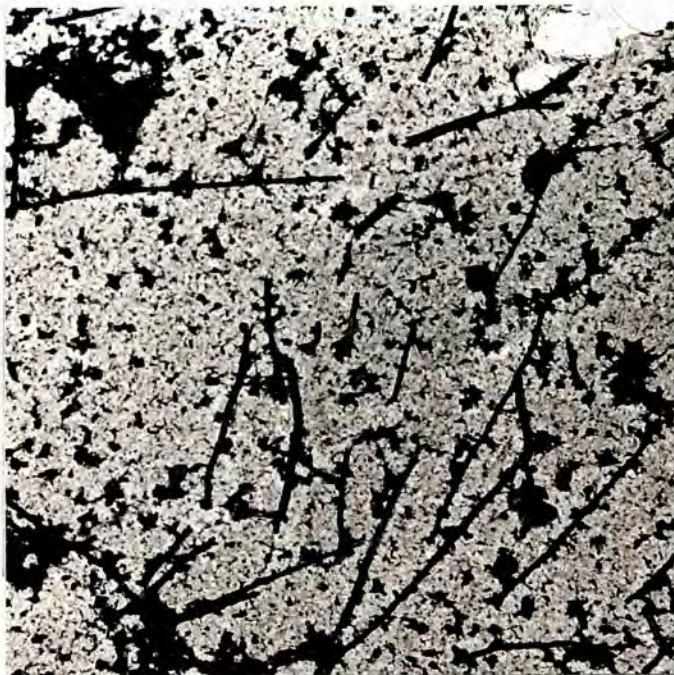


Fig. 4.15 B



Fig. 4.16 A Representative, highly magnified, micrograph of samples from lysed mitochondria supernatant, or synaptosol, extracted in the presence of EGTA and EDTA, without added  $MgCl_2$ . Incubation was carried out in microtubule-assembly buffer in the presence of glycerol. Under such conditions no filamentous structures were observed, but only ring structures, similar to those observed earlier on in  $S_1$  ( see fig. 4.9 A & 4.9 B), could be seen (see arrows). See also text, sec. 4.3.3.2. Magnification = 78,000.

Fig. 4.16 B Random field of negatively stained samples from the synaptosol fraction prepared incubated as described above. No microtubular structures were formed under such conditions. The ring structures seen are similar to those observed in  $S_1$  incubated in the presence of glycerol, with a diameter of about 20 nm (see text, sections 4.3.3.1 and 4.3.3.2. Magnification = 36,000





Fig.4.16 A



Fig.4.16 B





Fig. 4.17 A      Microtubular structures from samples of the synaptosomal fraction spiked with small volumes of  $S_1$ . Incubation was carried out in microtubule assembly buffer in the presence of 4 M glycerol. Very few microtubular structures were obtained similar in appearance to those resulting from  $S_1$  assembly (see fig. 4.9 A & B). See also text, section 4.3.3.3. Magnification = 11,000.

Fig. 4.17 B      Representative, highly magnified, microtubular structures from samples of the synaptosomal fraction incubated in the presence of  $S_1$  as described above. The width of these structures is between 20 & 30 nm. See also text, section 4.3.3.3. Magnification = 112,000.



Fig. 4.17A



Fig. 4.17B



Fig 4.18 A Representative, highly magnified, microtubular structures from samples of the synaptosomal fraction incubated in the presence of  $S_2$ . Incubation was carried out in microtubule assembly buffer in the presence of 4 M glycerol. Microtubular structures were very rare and not quite similar in appearance to those resulting from  $S_2$  assembly (see fig. 4.10 A & B). The width of the tubule was 25 - 30 nm. See also text, section 4.3.3.3. Magnification = 32,000.

Fig. 4.18 B Microtubular structures from samples of the synaptosomal fraction incubated in the presence of  $S_2$  as described above. Note the rare presence of such structures. See also text, section 4.3.3.3.  
Magnification = 15,000.



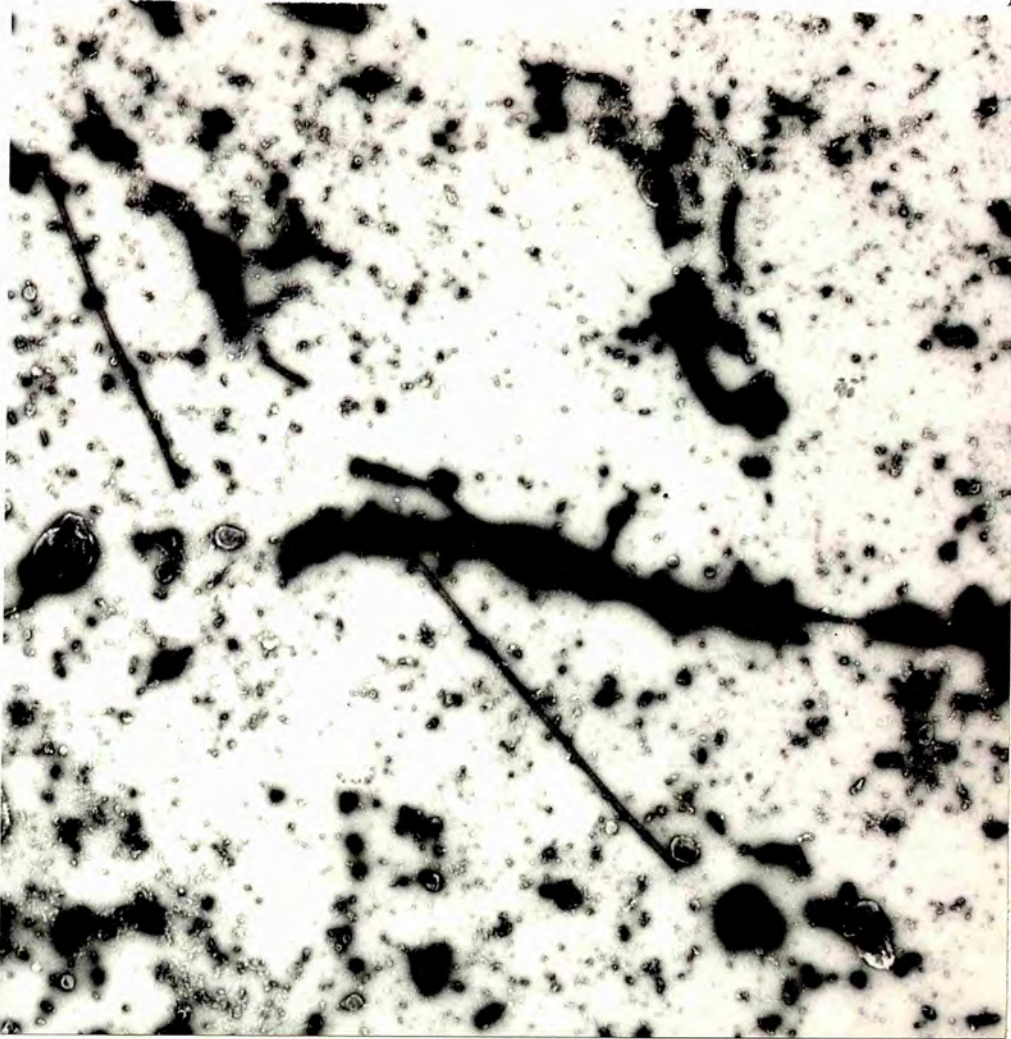


Fig. 4.18A

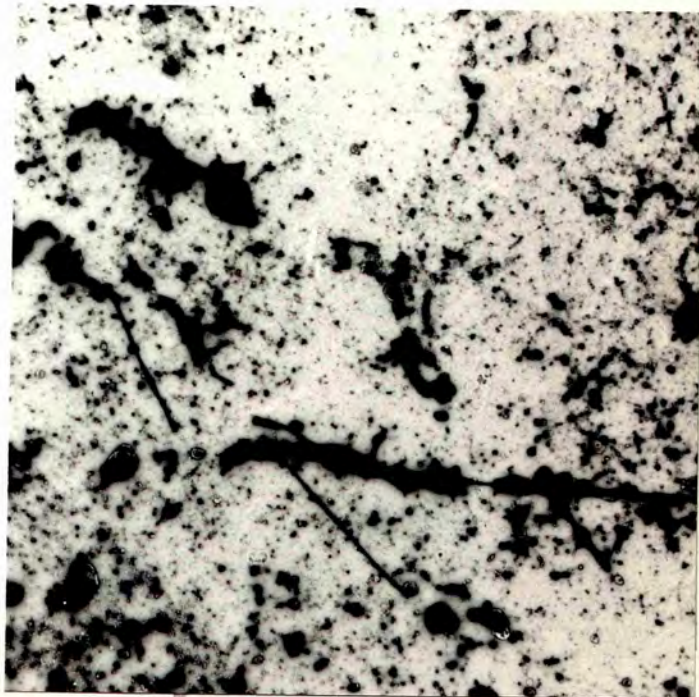


Fig. 4.18B



Fig. 4.19 A      Microtubular structures from samples of the diluted  $S_1$  fraction incubated in assembly buffer in the presence of 4 M glycerol. This preparation was used as a control for the synaptosol- $S_1$  combined fraction (fig. 4.17 A & B). Similarly to the spiked synaptosol preparation, it showed very few microtubular structures. (see text, section 4.3.3.3).  
Magnification = 11,000.

Fig. 4.19 B      Representative, highly magnified, microtubular structures from samples of the diluted  $S_1$  fraction incubated as described above. These structures were similar in appearance to the ones resulting from the incubation of non-diluted  $S_1$  fraction under similar conditions. (see fig. 4.9 A). Similar ring-like structures (ca. 20 nm in diameter) were also observed (see arrows). Magnification = 71,000.



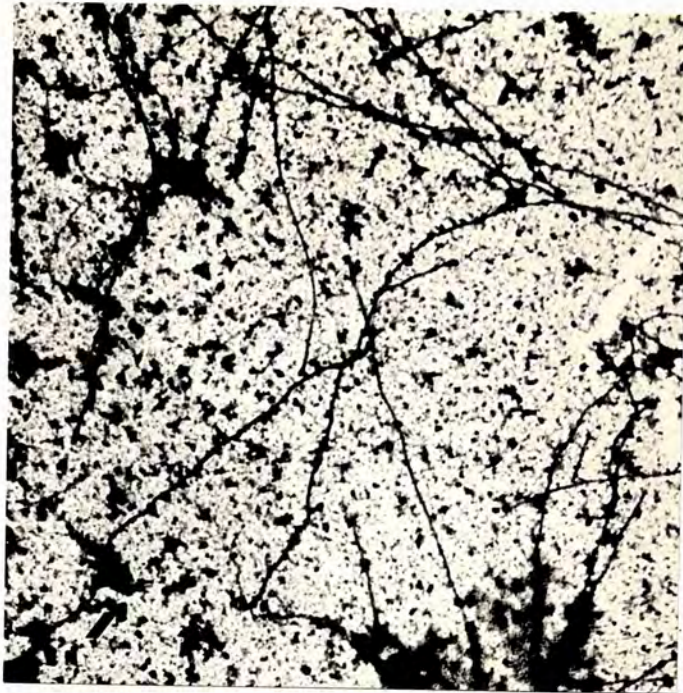


Fig. 4.19 A

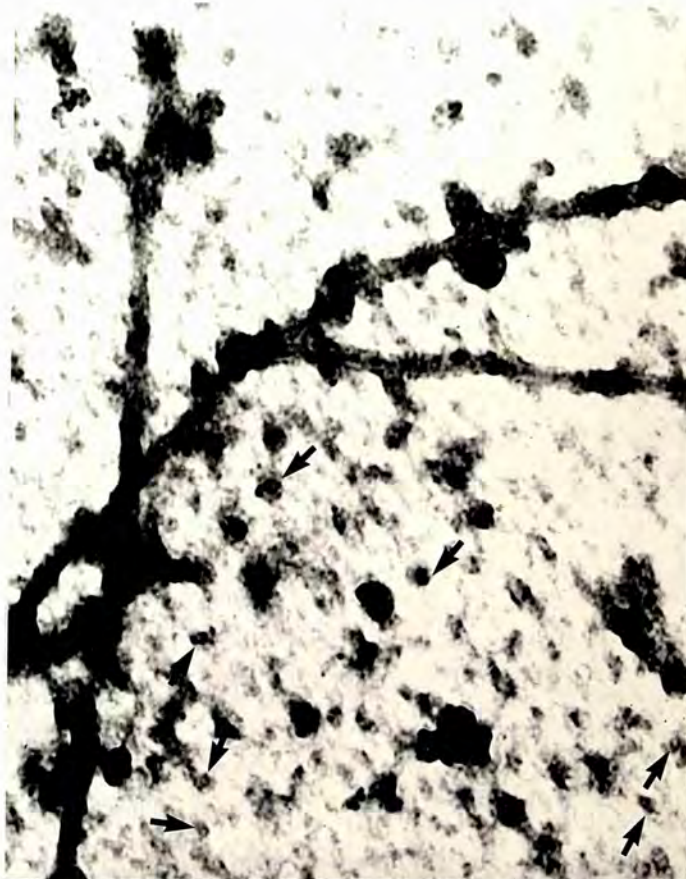


Fig. 4.19 B







Fig. 4.20 A Microtubular structures from samples of the diluted  $S_2$  fraction incubated in assembly buffer in the presence of 4 M glycerol. This preparation was used as a control for the synaptosol- $S_2$  combined fraction (fig. 4.18 A & B). Both the control and spiked synaptosol fractions showed very few microtubular structures (see text, section 4.3.3.3).  
Magnification = 29,000.

Fig. 4.20 B Representative, highly magnified, microtubular structures from samples of the diluted  $S_2$  fraction incubated as described above. These structures were few and dissimilar in appearance to those resulting from the incubation of concentrated  $S_2$  (see fig. 4.10 A). The present tubules have the same width (ca. 30 nm) as those observed in samples of synaptosol spiked with a small volume of  $S_2$ . See also text, section 4.3.3.3.  
Magnification = 50,000.



Fig.4.2 0 A



Fig.4.2 0 B

failing to see polymerized microtubules in synaptosomes is the low concentration of polymerization-competent tubulin present there. It can therefore be concluded that under the present experimental conditions little, if any putative tubulin present in the synaptosome fraction could be assembled in vitro.

#### 4.4 Discussion and conclusions

It may be concluded that while the synaptosome fraction appears to contain some tubulin, as judged by colchicine binding experiments and by electrophoretic analysis, the amounts present were insufficient to support the assembly process under the conditions tested. It is also possible that the anomalous behaviour of tubulin-like proteins in SDS gels reflects the presence of a form of tubulin different from that which can readily be polymerized into microtubules.

The failure to demonstrate microtubule assembly in synaptosome extracts, could also be due to losses, during the isolation procedure used, of endogenous accessory factors required for microtubule assembly. Such factors would probably be different from the HMW proteins, since these were present in the synaptosome, or from soluble factors present in fractions S<sub>1</sub> and S<sub>2</sub>, since these fractions were not effective in stimulating polymerization of synaptosome tubulin (see fig. 4.17 A & B and 4.18 A & B and text above).

This situation is not unique to synaptosomes since, for example, tubulin present in neuroblastoma cell extracts also failed to polymerize in vitro under similar conditions (Wiche and Cole, 1976), despite the presence of non-limiting amounts of tubulin in such extracts. Thus, a study of the conditions for obtaining polymerization of tubulin in synaptosome extracts should probably concentrate on preserving as much as possible

any synaptosome factors, possibly associated with microtubular structures, which might be required to demonstrate the assembly process in vitro.

For these reasons, conditions for using synaptosomal preparations in which microtubular structures are stabilized, as a starting material for obtaining assembly-competent tubulin, will be investigated in subsequent chapters.

Chapter V     The effect of microtubular stabilizing agents on the distribution of colchicine-binding protein in soluble and particulate fractions of rat brain

5.1     Introduction

5.2     Methods

5.3     Results

5.3.1     The effects of glycerol and DMSO

5.3.2     The effects of temperature

5.3.3     Comparison of buffer systems

5.3.4     Further studies on the distribution of CB protein in the stabilized homogenates

5.4     Conclusions

Chapter V    The effect of microtubular stabilizing agents on the distribution of colchicine-binding protein in soluble and particulate fractions of rat brain

5.1    Introduction

Early work on the assembly of microtubules by Shelanski et al. (1973) showed that the inclusion of 4 M glycerol in the assembly buffer markedly enhanced the polymerization of tubulin, and that the microtubules thus obtained were much more stable to low temperature and colchicine treatment. Removal of the glycerol resensitized the microtubules to cold and colchicine.

More recently, Filner et al. (1973) showed that the inclusion of 10% dimethylsulfoxide (DMSO) in the initial homogenizing medium led to the preservation of microtubular forms of tubulin during extraction of cow brains with phosphate buffer at room temperature. Microtubules sedimented from such extracts by centrifugation (at room temperature) could be preserved for several weeks after resuspension in a medium containing 10% DMSO and 8 M glycerol. Diluting such suspensions 10-fold with buffer alone led to an immediate disintegration of the microtubules, even at room temperature, while a two-fold dilution only made them labile in the cold. Furthermore, it was shown that the presence of these stabilizing agents did not by itself appear to induce any polymerization of the pre-existing pool of soluble tubulin in such extracts, even at room temperature.

Based on the above considerations, experiments were designed to determine the relative distribution of soluble, membrane-bound and microtubular forms of tubulin in homogenates prepared according to the proce-



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duro of Filner et al. (1973). In these experiments, colchicine binding was used to assay the tubulin contents of soluble and particulate fractions/obtained by centrifugation.

Soluble tubulin, in this context, refers to the colchicine-binding activity present in the initial supernatants obtained from homogenates prepared in the presence of stabilizers after spinning at room temperature (see fig. 5.1, fractions S<sub>2</sub> and PS<sub>2</sub>).

Microtubular tubulin is defined as that fraction of colchicine-binding activity which can be released in solution from particulates by extraction in stabilizer-free buffer at 4°C (i.e., fractions S<sub>3</sub> and PS<sub>3</sub> in fig. 5.1); colchicine-binding activity in the residual pellet will be referred to as membrane-bound tubulin (i.e., fractions P<sub>3</sub> and PP<sub>3</sub>).

## 5.2 Methods

Whole freshly excised rat brains were homogenized with three volumes of a solution (see Filner et al. 1973) containing 50% (v/v) glycerol, 10% (v/v) DMSO, 5 mM MgCl<sub>2</sub> and 10 mM sodium phosphate buffer, adjusted to pH 6.9 (microtubule stabilizing medium, MTK), by 10 up and down strokes at 2,000 rpm in a glass homogenizer fitted with a motor-driven Teflon pestle (clearance 0.08 - 0.13 mm). The homogenate was then fractionated according to the scheme shown in fig. 5.1. It was first centrifuged at 12,000 g for 15 min (at room temperature) to yield the primary fractions, P<sub>1</sub> and S<sub>1</sub>. S<sub>1</sub> was further spun for 60 min at 40,000 g (at 25-28°C) to give a soluble fraction (S<sub>2</sub>) containing soluble tubulin and a pellet, P<sub>2</sub>, containing both stabilized microtubules and membranes. Fraction P<sub>2</sub> was then resuspended in a stabilizer-free, microtubule assembly buffer (to half the volume of S<sub>1</sub>), which contains 0.1 M MES buffer, 1 mM EGTA, 0.5 mM

MgCl<sub>2</sub> and 1 mM GTP, adjusted to pH 6.9 (see also Chapter II, section 2.2.1), and kept on ice for 20 min to promote the depolymerization of microtubules. The suspension was then spun at 100,000 g for 30 min at 4°C to yield a soluble microtubular tubulin fraction (S<sub>3</sub>) and a residual membrane fraction (P<sub>3</sub>).

All fractions were tested for colchicine-binding activity after diluting them in the cold with 2 volumes of the above assembly buffer. One volume of sample solution was added to four volumes of incubation buffer (10 mM sodium phosphate buffer containing 5 mM MgCl<sub>2</sub> and adjusted to pH 6.5) and the reaction was started by the addition of 50 μl of (<sup>3</sup>H) colchicine (0.2 Ci/mM; final concentration 50 μM) and terminated in an ice-bath after 2 hours incubation at 37°C (see Chapter II, section 2.3.1). Since DMSO and glycerol were only present in fraction S<sub>2</sub>, they were added to the reaction mixture for all the other fractions to give the same final concentration (1% and 0.3 M, respectively) so that the binding reaction would take place under the same conditions in all samples.

Colchicine-binding activity was determined in triplicate by the DE 81 filter disc assay (see Chapter II, section 2.3.2).

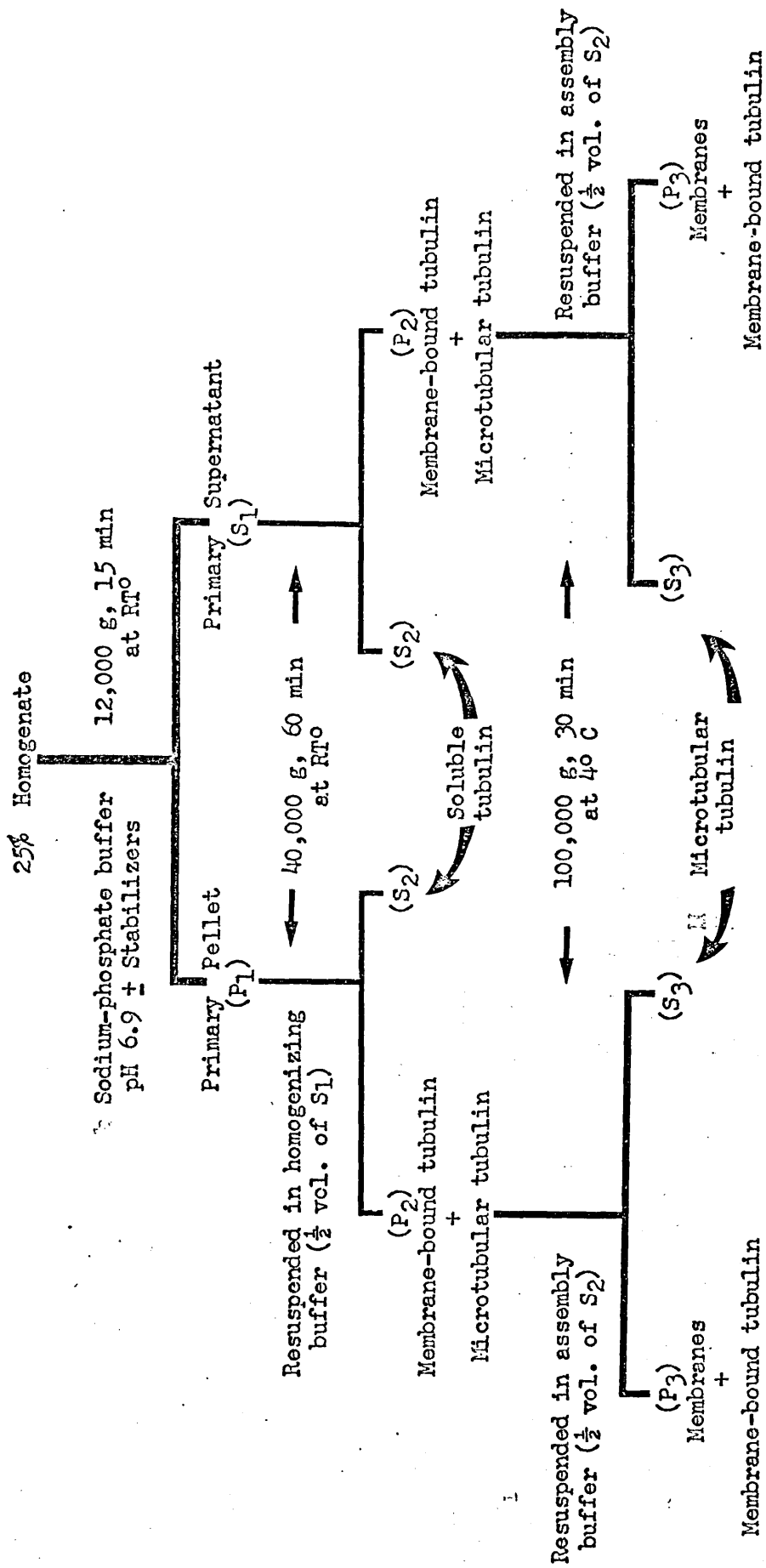
The protein content of the fraction was determined by Folin-Lowry method (see Chapter II, section 2.6).

Finally, additional experiments were carried out to determine the contribution of fraction P<sub>1</sub> to the total colchicine-binding activity present in the initial homogenate. The distribution of colchicine-binding activity was also determined in subfractions of P<sub>1</sub> that were derived using the method employed to fractionate the primary fraction, S<sub>1</sub>.



Fig. 5.1 Diagrammatic scheme for the extraction of different forms of tubulin from whole rat brain homogenate.

Soluble, (S<sub>2</sub>), microtubular, (S<sub>3</sub>) and membrane-bound, (P<sub>3</sub>) tubulin fractions were extracted from whole brain homogenate according to the method of Filner et al. (1973). Fraction (P<sub>3</sub>), also contained a high proportion of membrane components (see text, section 5.2).



**Fig. 5.1**

### 5.3 Results

#### 5.3.1 The effects of glycerol and DMSO

It is evident from the results shown in table 5.1 that a substantial increase in the proportion of colchicine binding (CB) recovered in the particulate fraction  $P_2$  was found in homogenates prepared in the presence of both glycerol and DMSO. Since most of this activity could be subsequently released under conditions favoring depolymerization of microtubules (i.e., stabilizer-free buffer in the cold) as seen from the distribution and specific activity (S.A.) values for CB (see fraction  $S_3$ , table 5.1, parts A & B), it is probable that fraction  $S_3$  contains tubulin derived from microtubules preserved during the initial homogenization (see also below). These results confirm and extend the preliminary observations of Filner et al.

However, the CB activities in the membrane fraction ( $P_3$ ) was reproducibly found to be higher in material derived from homogenates containing glycerol alone as compared to that from homogenates prepared in the presence of both DMSO and glycerol (compare S.A. values for fraction  $P_3$  in parts A & B of table 5.1). This may be correlated with the slightly higher value of CB activity found in the  $S_3$  fraction derived from homogenates prepared in the presence of both stabilizing agents.

Thus, it would appear that DMSO present during initial homogenization affects the state of CB protein associated with the  $P_2$  fraction (i.e., containing membranes and microtubules), as reflected by the observed differences in the solubility of  $P_2$ -associated CB protein during subsequent extraction of  $P_2$  in the cold in stabilizer-free medium. This may reflect differences, specifically induced by DMSO, in the nature and

extent of microtubule-membrane interactions (see discussion).

In the present experiments, it is also apparent that a similar enrichment of CB activity was found in the putative microtubule-containing fractions ( $P_2$  &  $S_3$ ) when only glycerol was included as a stabilizing agent, as can be seen from the specific activity values shown in table 5.1 (cf. parts A & B).

Furthermore, it is evident that fraction  $S_3$ , prepared from homogenates containing one or both stabilizing agents, was considerably enriched in microtubular protein, since the CB specific activity values for this fraction were several-fold higher than those seen in all other fractions examined. Furthermore, it is also clear from the data shown in part C of table 5.1, that this enrichment was much less apparent in the corresponding ( $S_3$ ) fraction prepared in the absence of stabilizing agents.

### 5.3.2 The effects of temperature

Evidence that microtubules can be preserved in brain homogenates prepared at room temperature in the presence of stabilizing agents was presented above. However, the relative contribution of stabilizing agents and of temperature conditions to the preservation of microtubules could not be assessed.

From the previous results shown in table 5.1 (part C), it can be seen that a slight enrichment in CB activity was found after solubilization in the cold of the putative microtubular containing fraction ( $P_2$ ) when this fraction was prepared (at room temperature) in the absence of added stabilizing agents. However, it is well known that very little

CB activity can be extracted from brain particulate fractions by various buffers when these fractions are prepared in the cold (see Lagnado et al. 1971). Therefore, it can be inferred that the relatively small but significant CB activity that was solubilized from fraction  $P_2$ , when this was prepared at room temperature in the absence of stabilizing agents (40% as compared to 60%; see table 5.1, part C) probably represents, in part at least, microtubule-derived protein.

Further experiments specifically designed to test the effect of temperature during the initial homogenization clearly showed that microtubules were preserved to a much lesser extent at  $4^{\circ}\text{C}$  than at room temperature, even when stabilizing agents were included in the initial homogenization medium (see table 5.3). Indeed, when homogenates were prepared and centrifuged at  $4^{\circ}\text{C}$ , 90% of the CB activity present in the initial supernatant fraction ( $S_1$ ) remained in solution (in fraction  $S_2$ ), after the further centrifugation step to pellet the 'microtubular-membrane' fraction ( $P_2$ ), as compared to values of 70% found at room temperature (see tables 5.2 & 5.3). These differences are due to a selective redistribution of CB activity as can be seen from the RSA values shown in table 5.3.

It is concluded that the presence of stabilizing agents by itself is not a sufficient condition for the preservation of microtubules during the initial homogenization even though, once formed, microtubular structures can be preserved at  $4^{\circ}\text{C}$  or indeed at  $-10^{\circ}\text{C}$  in the presence of stabilizing agents (see below). It is inferred that the stabilizing agents did not increase the content of microtubules through a direct effect in promoting microtubular assembly from soluble tubulin pools, but rather exerted a stabilizing influence on formed microtubules already present in the tissue before extraction. It can be concluded, moreover, that



the presence of these agents could not prevent the rapid cold-induced disassembly of microtubules which presumably occurred during the time taken for homogenizing the tissue at 4°C. These results do not therefore contradict the observations of Filner et al. and my own unpublished observations that pelleted microtubules resuspended in the presence of glycerol and DMSO can be preserved for several days at temperature ranging between 4°C and -10°C. Previous work has also shown that colchicine-binding activity of preparations stored under these conditions can also be preserved for the same period of time (Lagnado, Tan and Reddington, unpublished observations).

### 5.3.3 Comparison of buffer systems

In the above experiments, microtubule assembly buffer (see section 5.2) was employed to solubilize microtubular protein while phosphate buffer was used during the initial homogenization of the tissue. Further experiments were carried out to investigate whether the initial homogenization of the tissue in a buffer favoring microtubular assembly might alter the yield of CB activity in microtubule-containing fractions derived from tissue homogenates containing microtubule-stabilizing agents.

The results shown in table 5.2 indicate that the CB activity in the microtubular fractions derived from tissue extracted in assembly buffer was up to 50% higher than that found for the corresponding fractions derived from phosphate-buffered homogenates (compare specific activity values for S<sub>3</sub> in tables 5.2 and 5.1, parts A & B).

However, similar differences were also apparent for the mother fraction (P<sub>2</sub>) and for the soluble tubulin fraction (S<sub>2</sub>). On the other hand, the CB activities in the membrane fractions were very similar using

the two types of buffer conditions when DMSO and glycerol were used as stabilizing agents (compare values for  $P_3$  in tables 5.1 & 5.2, parts A and B), whereas the general increase in CB activity observed, using assembly buffer, was also apparent in the membrane fractions when glycerol alone was used as a stabilizing agent. It is interesting to note that the relative increase observed in the CB activity of fraction ( $P_3$ ) when this was derived from DMSO-containing homogenates (see table 5.2), was confirmed in the present experiments (compare values for  $P_3$  in parts A & B of table 5.2).

It seems, therefore, that CB activity is generally better preserved throughout the various fractions when the initial homogenization is carried out using microtubular assembly buffer rather than the phosphate buffer employed by Filner et al. However, it cannot be deduced, from the present experiments if the differences observed are related, per se, to the polymerization-promoting properties of the buffer (which could affect the physical state of the protein in such a way as to preserve colchicine-binding sites), since it is not known whether factors which influence the assembly-competence of tubulin are in any way related to those which preserve the CB properties of tubulin. Since calcium ions do not apparently affect CB activity (see Lyons, C., 1977), it seems unlikely that the differences observed with the two buffer systems are due to the inclusion of EGTA in the assembly buffer. On the other hand, it is well known that GTP preserves tubulin in respect of both its CB activity and its competence for polymerization (see Chapter I, section 1.8). Since GTP was not included in the phosphate buffer system of Filner et al., it is tentatively concluded that GTP was mainly responsible for the differences in CB activity found in the two series of experiments described above.



Table 5.1    Distribution of colchicine-binding activities in different tubulin-containing fractions from rat brain homogenized in sodium phosphate buffer in the presence of different stabilizing agents.

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Results shown are means of triplicate determinations from three separate experiments ( < 10% difference between experiments). Values in the first two columns are given as a percentage of protein or binding activity recovered from mother fractions: S<sub>1</sub> and P<sub>2</sub>. PSA, or relative specific activity, = % bound c.p.m. recovered/% protein recovered. Binding activities were assayed radiometrically as described in Chapter II, section 2.3.2. See more details in text, section 5.3.1.


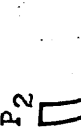
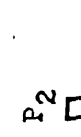
Experimental Conditions	Fraction	Description	Colchicine Distribution (%)	Protein Distribution (%)	R.S.A	Specific activity cpm/ $\mu$ g. protein
A + DMSO + Glycerol	S <sub>2</sub>	Soluble tubulin	75.0	83.2	0.9	25
	P <sub>2</sub> 	Membrane-bound tubulin + Micotubular tubulin	25.0	16.8	1.5	41
		Membrane-bound tubulin	37.5	77.0	0.5	15
	S <sub>3</sub>	Micotubular tubulin	62.5	23.0	2.7	109
B - DMSO + Glycerol	S <sub>2</sub>	Soluble tubulin	76.0	83.7	0.9	29
	P <sub>2</sub> 	Membrane-bound tubulin + Micotubular tubulin	24.0	16.3	1.5	45
		Membrane-bound tubulin	37.0	70.4	0.5	23
	S <sub>3</sub>	Micotubular tubulin	63.0	29.6	2.1	95
C - DMSO - Glycerol	S <sub>2</sub>	Soluble tubulin	84.0	87.0	1.0	20
	P <sub>2</sub> 	Membrane-bound tubulin + Micotubular tubulin	16.0	13.0	1.2	25
		Membrane-bound tubulin	61.5	69.0	0.9	22
	S <sub>3</sub>	Micotubular tubulin	38.5	31.0	1.2	31

Table 5.1



Table 5.2      Distribution of cholchicine-binding activities in different tubulin-containing fractions from rat brain homogenized in MES buffer system in the presence of different stabilizing agents.

Results shown are means of triplicate determinations from three separate experiments (< 10% difference between experiments). Values in the first two columns are given as a percentage of protein or binding activity recovered from mother fractions: S<sub>1</sub> and P<sub>2</sub>. RSA, relative specific activity, = % bound c.p.m recovered/% protein recovered. Binding activities were assayed radiometrically as described in Chapter II, section 2.3.2. See also text, section 5.3.3.

Experimental Conditions	Fraction	Description	Colchicine Distribution (%)	Protein Distribution (%)	R.S.A	Specific activity cpm/ $\mu$ g protein
A + DMSO + Glycerol		Soluble tubulin	70.0	80.0	0.9	38
		Membrane-bound tubulin + Microtubular tubulin	30.0	20.0	1.5	69
		Membrane-bound tubulin	20.5	66.5	0.3	18
		Microtubular tubulin	79.5	33.5	2.4	138
B - DMSO + Glycerol		Soluble tubulin	76.0	82.6	0.9	45
		Membrane-bound tubulin + Microtubular tubulin	24.0	17.4	1.4	68
		Membrane-bound tubulin	29.0	69.3	0.4	34
		Microtubular tubulin	71.0	30.7	2.3	156

Table 5.2



Experimental Conditions	Fraction	Description	Colchicine Distribution (%)	Protein Distribution (%)	R.S.A	Specific activity cpm/ $\mu$ g protein
A	+ DMSO	S <sub>2</sub> Soluble tubulin	68.2	79.5	0.9	5
	+ Glycerol RT	P <sub>2</sub> { Membrane-bound tubulin + Microtubular tubulin	31.8	20.5	1.6	10
B	+ DMSO	S <sub>2</sub> Soluble tubulin	92.0	84.0	1.1	24
	+ Glycerol 4° C	P <sub>2</sub> { Membrane-bound tubulin + Microtubular tubulin	8.0	16.0	0.5	5

**Table 5.3** Distribution of colchicine-binding activities in soluble and microtubular tubulin fractions from rat brain homogenized in MES buffer in the presence of stabilizing agents, at different temperatures (room temperature and 4°C).

Results shown are means of triplicate determinations from two separate experiments (<10% difference between experiments). Values in the first two columns are given as a percentage of protein or binding activity recovered from the corresponding fraction. RSA, relative specific activity, = % bound c.p.m. recovered/% protein recovered. See also text, section 5.3.2.

5.3.4 Further studies on the distribution of CB protein in the stabilized homogenates

During the course of this work, the distribution of CB activity present in the initial homogenate was investigated in fractions derived from a low-speed extract (fraction  $S_1$ ) containing the stabilized microtubules. Further experiments were undertaken to determine the extent to which microtubular forms of tubulin might have been left in the low-speed pellet fraction  $P_1$  (see fig. 5.1) that was normally discarded.

To investigate this possibility, homogenates were prepared in assembly buffer containing 10% (v/v) DMSO and 50% (v/v) glycerol and the relative distribution of CB activity in the  $S_1$  and  $P_1$  primary fractions, as well as in their respective subfractions was determined.  $S_1$  was fractionated as previously described, as was the  $P_1$  fraction, following its resuspension at room temperature in a volume of homogenizing buffer (containing stabilizers) equivalent to that of  $S_1$ .

As can be seen from the data summarized in tables 5.4 and 5.5, most of the CB activity and protein present in the resuspended  $P_1$  fraction was recovered in the pellet obtained by further centrifugation at room temperature ( $P_2$ ), even though no enrichment of CB was found in this fraction (cf. SA & RSA values for  $P_2$  and  $S_2$ , table 5.5). This was in marked contrast to the results found for the  $P_2$  fraction derived from  $S_1$  in this (table 5.4) and in previous experiments (tables 5.2, part A; & 5.3, part A).

Furthermore, it was found that resuspension of the pellet ( $P_2$ ) prepared at room temperature from the particulate ( $P_1$ ) in stabilizer-free buffer resulted in very little solubilization of CB activity ( $S_3$  table 5.5): most of the activity remained membrane-bound (cf. data in table 5.4 & 5.5).



Table 5.4     Distribution of colchicine-binding activities in soluble and particulate fractions of rat brain homogenized in MES buffer system in the presence of stabilizing agents.

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Results shown are means of triplicate determinations from three separate experiments ( < 10% difference between experiments). Values in the first two columns are given as a percentage of protein or binding activity recovered from mother fractions: S<sub>1</sub> and P<sub>2</sub>. RSA, relative specific activity, = % bound c.p.m. recovered/% protein recovered. See also text, section 5.3.4.

Mother Fraction	Sub-Fraction	Description	Colchicine Distribution (%)	Protein Distribution (%)	R.S.A	Specific activity cpm/ $\mu$ g protein
S <sub>1</sub>	S <sub>2</sub>	Soluble tubulin	70.0	80.0	0.9	38
	P <sub>2</sub>	Membrane-bound tubulin + Microtubular tubulin	30.0	20.0	1.5	69
		Membrane-bound tubulin	20.5	66.5	0.3	18
	S <sub>3</sub>	Microtubular tubulin	79.5	33.5	2.4	138

Mother Fraction	Sub-Fraction	Description	Colchicine Distribution (%)	Protein Distribution (%)	R.S.A	Specific activity cpm/ $\mu$ g protein
P <sub>1</sub>	S <sub>2</sub>	Soluble tubulin	18.5	9.9	1.9	43
	P <sub>2</sub>	Membrane-bound tubulin + Microtubular tubulin	81.5	90.1	0.9	22
		Membrane-bound tubulin	88.3	89.0	1.0	16
	S <sub>3</sub>	Microtubular tubulin	11.7	11.0	1.1	17

Table 5.4

Type of binding Protein	S <sub>1</sub>		P <sub>1</sub>		Colchicine Distribution (%)	R.S.A	Protein Distribution (%)	Colchicine Distribution (%)	R.S.A	Colchicine Distribution (%)
	Colchicine Distribution (%)	Protein Distribution (%)	Colchicine Distribution (%)	Protein Distribution (%)						
(S <sub>2</sub> ) Soluble tubulin	69.0	80.0	0.9	0.9	24.0	2.4	10.0	2.4	2.4	44.0
(S <sub>3</sub> ) Microtubular tubulin	23.0	6.0	3.8	3.8	9.0	1.0	9.0	1.0	1.0	15.0
(P <sub>3</sub> ) { Membranes + Membrane-bound tubulin	8.0	14.0	0.6	0.6	67.0	0.8	81.0	0.8	0.8	41.0

**Table 5.5** Total distribution of colchicine-binding activities the soluble, microtubular and membrane-bound tubulin-containing fractions from rat brain homogenized in MES buffer system in the presence of stabilizing agents.

Results shown are summarized from tables 5.2 and 5.4. Values in columns 1, 2, 4 and 5 are given as a percentage of protein or binding activity recovered from mother fractions S<sub>1</sub> and P<sub>1</sub>. Values in column 7 are given as a percentage of binding activity recovered from the total homogenate. See also text, section 5.3.4.

Thus, it is concluded that the bulk of the CB activity of fraction  $P_1$  was associated with the membrane fraction. Furthermore, the data summarized in table 5.6 indicate that microtubular tubulin (= CB in  $S_3$ ) contributed less than 10% of the total CB activity of the low-speed particulate ( $P_1$ ), as compared with values more than 20% found for the microtubular fraction derived from  $S_1$ .

As can be seen from the data summarized in table 5.6, about 2/3 of both the soluble and microtubular tubulin fractions present in homogenates were recovered in the low-speed soluble fraction,  $S_1$ , whereas the bulk of the membrane-bound CB activity was associated with the low-speed particulate fraction, ( $P_1$ ). Finally, it can be seen that only about 15% of the total CB activity present in stabilized homogenates originates from microtubules; the remainder was distributed fairly evenly between the soluble and the membrane fractions.

#### 5.4 Conclusions

From the data described in this chapter, it can be inferred that about 15% of the colchicine-binding activity can be sedimented at room temperature from homogenates prepared in the presence of glycerol and DMSO. Furthermore, this fraction could be solubilized in the cold in stabilizer-free buffer. Thus, we are dealing with a labile form of aggregatable material which presumably presents microtubular form of assembly-competent tubulin.

Indeed, additional experiments carried out subsequently by other members of this laboratory have demonstrated the polymerization competence of the fraction referred to in this chapter as microtubular tubulin (i.e., fraction  $S_3$ ). Electron microscopy showed the presence of numerous micro-

tubules in samples of  $S_3$  which had been further incubated at  $37^{\circ}\text{C}$  in the presence of added GTP, with or without glycerol (see fig. 5.2). During these experiments, it was also shown that tubulin comprised about 80% of the protein present in the  $S_3$  fraction, as judged by SDS-PAGE analysis.

In contrast, attempts to polymerize microtubules from the fraction referred to as soluble tubulin (i.e., fraction  $S_2$ ) were unsuccessful under a variety of experimental conditions despite the fact that this fraction was rich in CB activity (see sections 5.3.1 & 5.3.3) and contained a significant amount of tubulin detectable by SDS-PAGE.

Thus, an additional conclusion from the work described above would be that two forms of soluble tubulin are present in fractions  $S_2$  and  $S_3$  in brain homogenates prepared under the conditions described, only one of which, that recovered in  $S_3$ , apparently originates from cold-labile microtubules.

The significance of the membrane-bound CB activity which was described in this work remains to be elucidated. It could, for example, represent cold-stable non-microtubular aggregates of tubulin, non-tubulin membranous components capable of binding colchicine as well, perhaps, as some tubulin-like protein present in membranes. These various possibilities will be discussed later (Chapter VII).

Having established suitable conditions to assay the different forms of tubulin - and in particular, tubulin originating from microtubules - in crude brain extracts, the possibility of extending this approach to the study of microtubular proteins in defined subcellular fractions of brain was investigated, as will be described in the next chapter.



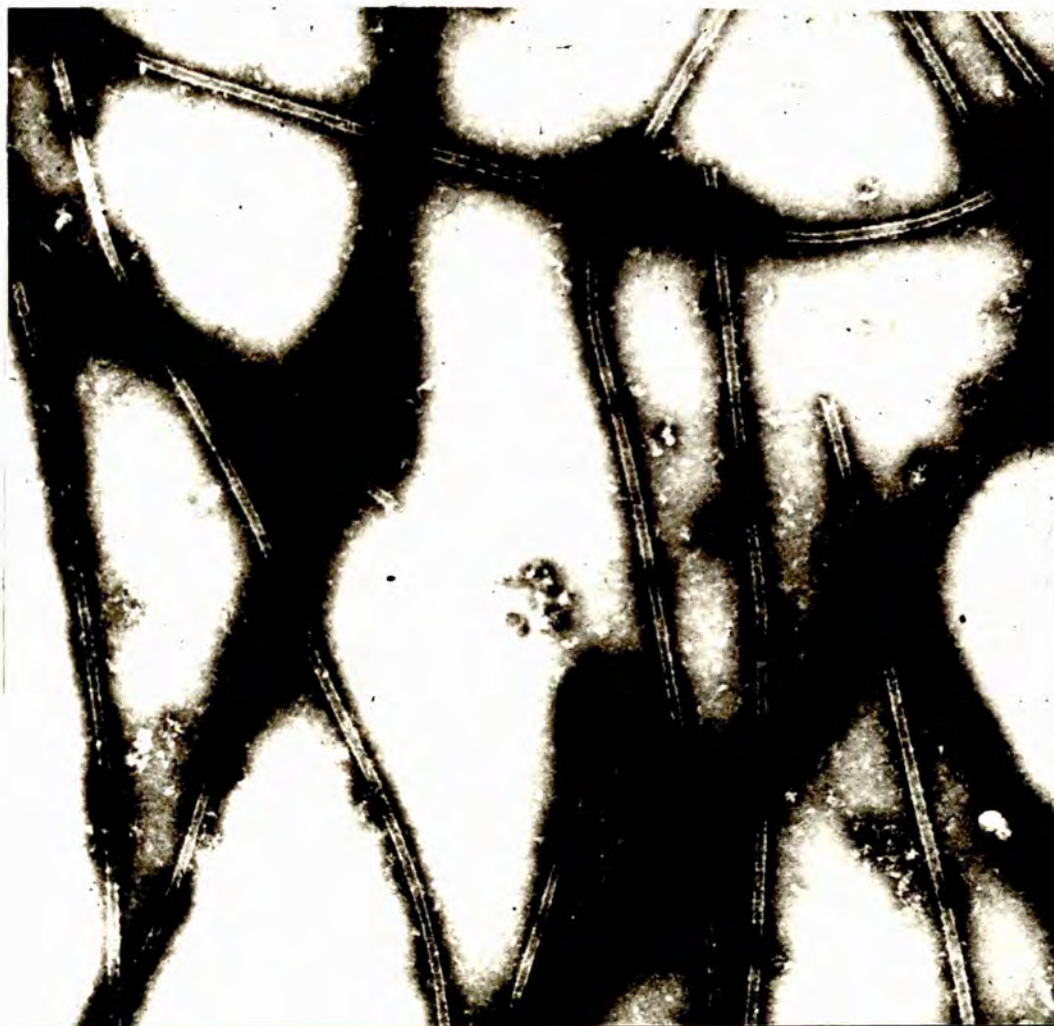


Fig. 5.2 Representative microtubules from a microtubular tubulin-containing fraction,  $S_3$ , separated by the Shelanski method (see Chapter II, section 2.2.1) and incubated in assembly buffer in the presence of 4 M glycerol. For details see text, section 5.4). Courtesy of Dr L.P. Tan. Magnification = 100,000.

Chapter VI      Studies on the stabilization and polymerizability  
of chick brain synaptosomal microtubular protein

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Chapter VI    Studies on the stabilization and polymerizability  
of chick brain synaptosomal microtubular protein

6.1    Introduction

Preliminary attempts to reassemble microtubules from conventionally prepared soluble extracts of synaptosomes were unsuccessful (see Chapter IV), even though the presence of tubulin as a major component in such extracts was evident from both electrophoretic analysis (Chapter IV) and binding studies using antimicrotubular drugs (see Chapter III).

This could be due to the relatively low protein content of synaptosomal extracts investigated since it is well known that the polymerization of tubulin does not occur below a so-called "critical concentration" of the protein (see Chapter IV). Another possibility is that tubulin itself, or microtubule-associated proteins required for assembly (see Chapter I), may have been altered, and/or degraded, during the procedures employed to prepare synaptosomal extracts in such a way as to limit the pool of assembly-competent microtubular protein, thus reducing still further the concentration of 'active' tubulin for polymerization.

The recent morphological observations of Hajós (1976) indicate that abundant microtubular structures can be seen in rapidly isolated synaptosome preparations (Hajós, 1975); these are **preincubated** at room temperature in isotonic saline media (Hajós, 1976) before fixing and staining the particles

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for electron microscopy. No tubules could be seen following preincubation at 4 °C. From this, it can be concluded that microtubules present in nerve endings are very sensitive to temperature and ionic conditions, similarly to microtubules seen within axonal or dendritic processes. This suggests that lysis and subsequent extraction of synaptosomes at room temperature, following a brief preincubation in physiological saline (also at room temperature) might allow some preservation of assembly-competent microtubular protein subunits as well as of microtubules, which could be sedimented during centrifugation of the crude lysed extracts. Furthermore, in view of the stabilizing effects of DMSO and glycerol on microtubules (see Chapter V), it seemed worthwhile to investigate whether their inclusion during the preparation of synaptosol extracts in this way might facilitate the isolation of assembly-competent tubulin.

## 6.2 Methods

### 6.2.1. Subcellular fractionation

The first step in the present subcellular fractionation experiments involved the isolation of synaptic terminals or synaptosomes. This was accomplished by following the method of F. Hajós (see fig. 6.1 A). Once obtained, such synaptosomes were incubated for 30 min at room temperature in an isotonic saline solution containing 80 mM NaCl, 5 mM KCl, buffered with 10 mM Tris HCl pH 7.4. This incubation is mainly aimed at restoring the microtubular formations described by F. Hajós (unpublished).

The following steps (see fig. 6.1 B) involved an extension of this method, with view to isolating stabilized microtubular structures. For this purpose, the synaptosomes were pelleted and resuspended in a 4-fold diluted assembly buffer (containing Mes, GTP, EGTA,  $MgCl_2$ ; see Chapter II, section 2.2.1) to help in the lysis process which was performed, at room temperature, over a period of 20 min with intermittent sonication (3x 10 sec in an MSE 150 watt ultrasonic disintegrator, operated at medium power and optimum amplitude). The lysed material was gently homogenized by hand and incubated at  $37^{\circ}C$  for 15 min in the presence or absence of a microtubule stabilizing agent (i.e., 10% (v/v) DMSO or 4 M glycerol; see Chapter V), and centrifuged at 38,000 g for 30 min at room temperature to yield a putative microtubule-containing membrane pellet (=  $SP_1$ ) and a supernatant (=  $SS_1$ ), containing soluble and presumably non-polymerizable tubulin. The pellet was then resuspended in assembly buffer (standard concentration) in the absence of DMSO or glycerol, and kept on ice to promote microtubule depolymerization, and any microtubular subunits that may have been released by this process were then separated from the membranes by centrifugation at 100,000 g for 60 min at  $4^{\circ}C$ . The cold-soluble extracts ( $SS_2$ ) and the cold-insoluble pellet ( $SP_2$ ) were then used for further investigations, i.e. colchicine-binding activity, polymerizability and gel electrophoresis. Fraction  $SP_2$  would presumably consist of membranes and possibly, cold-insoluble forms of tubulin aggregates, while fraction  $SS_2$  is assumed to be enriched in polymerizable tubulin derived from stabilized synaptosomal microtubules.

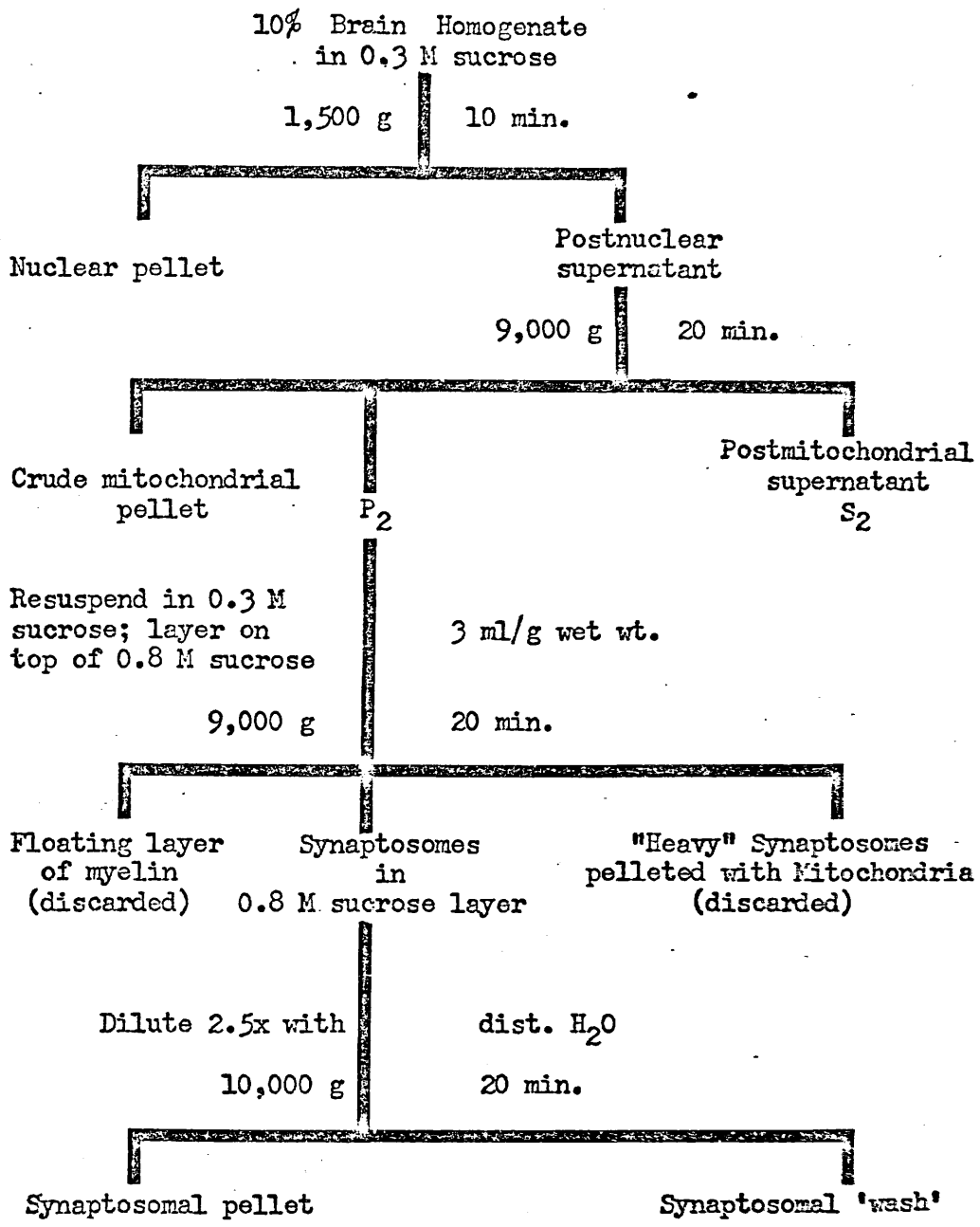


Fig. 6.1 A Diagrammatic scheme for the purification of synaptosomes from chick brain according to the Hajos method (1975). See also text, section 6.2.1.

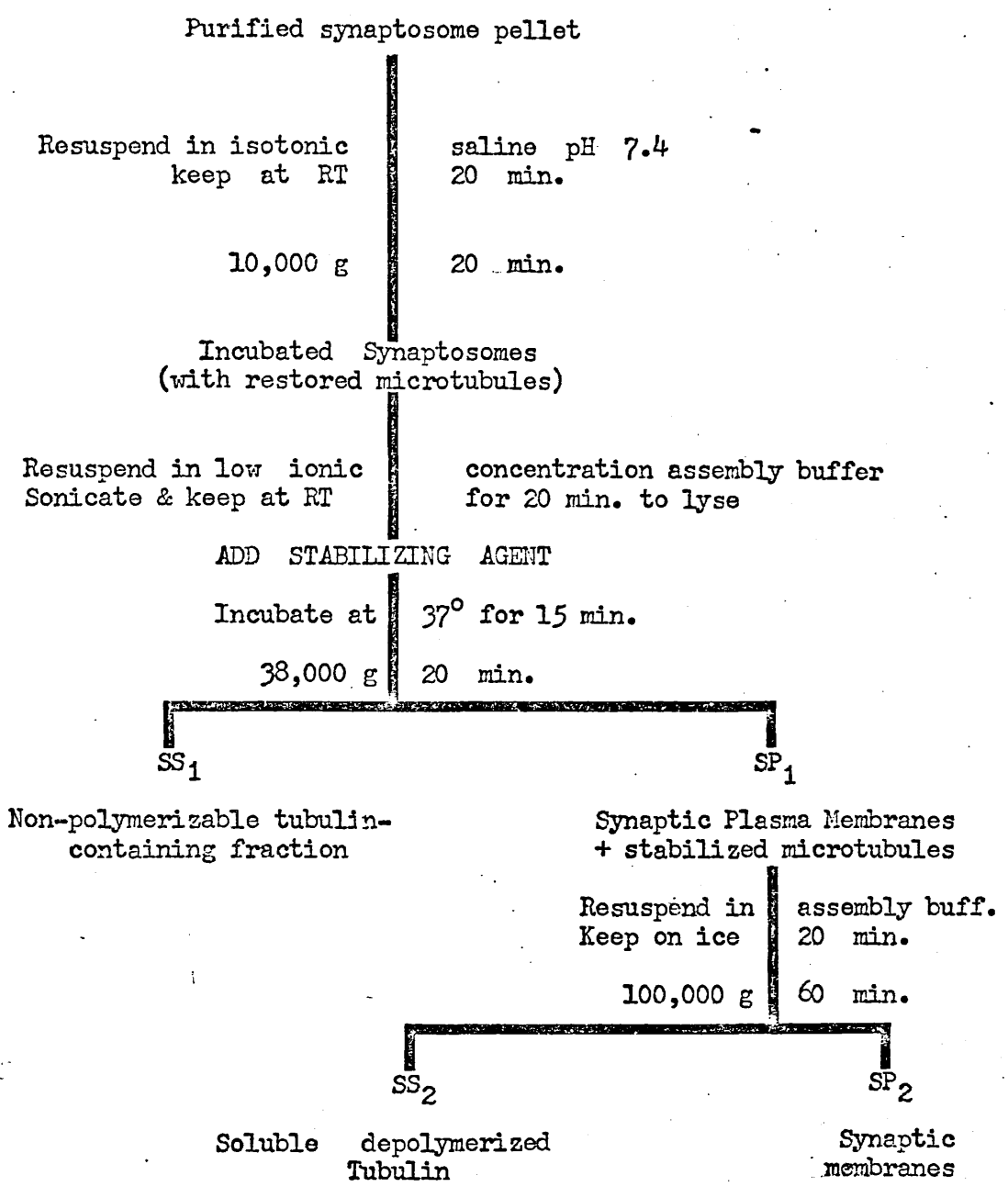


Fig. 6.1 B Further fractionation of chick brain synaptosomes, purified by the Hajos method, to extract soluble synaptosomal tubulin. See text, section 6.2.1.

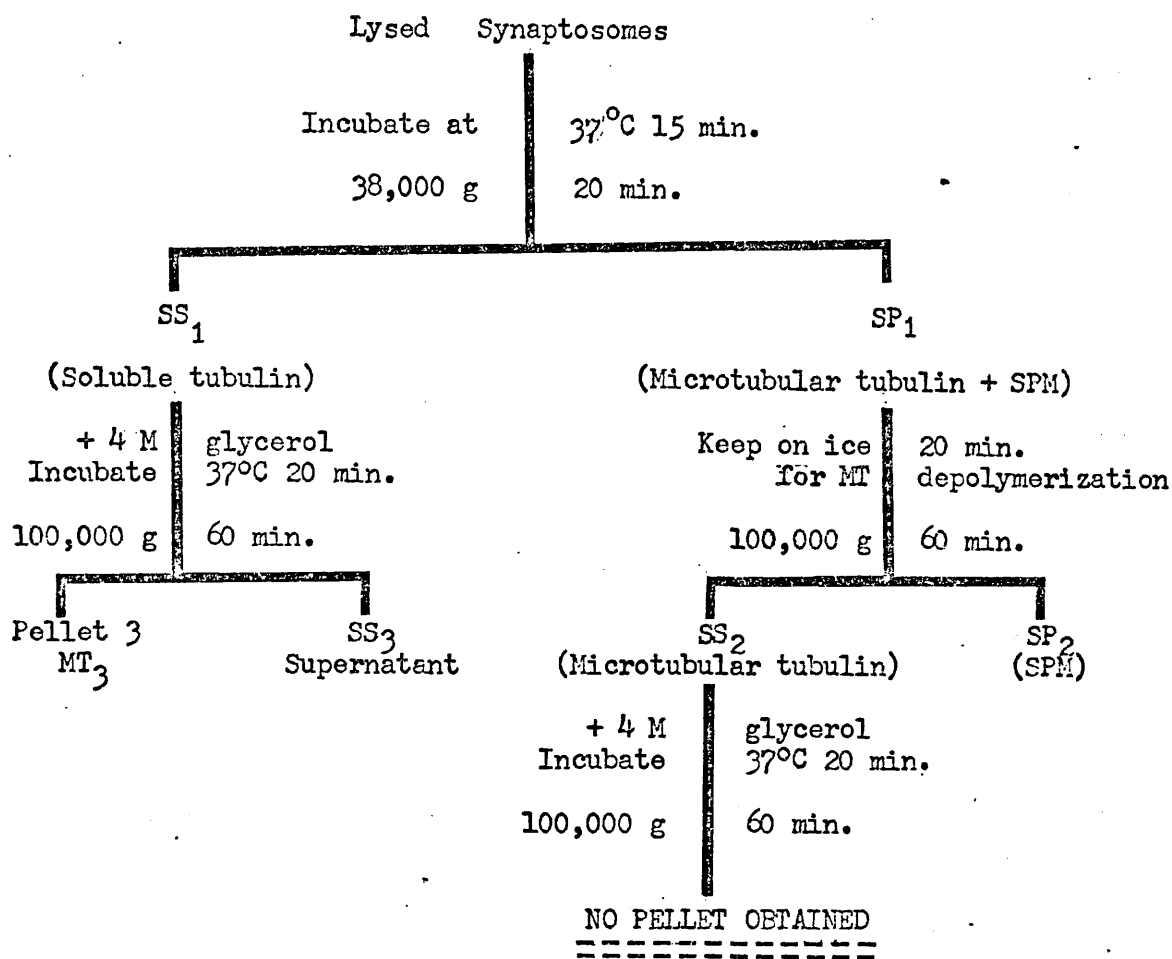


Fig. 6.2 Scheme for the assembly of soluble, SS<sub>1</sub>, and microtubular, SS<sub>2</sub>, tubulin from chick brain synaptosomes purified by the method of Hajos. For MT<sub>3</sub> gel see text, section 6.3.3 (SS<sub>1</sub>).



### 6.2.2 Colchicine-binding activity

Aliquots from the synaptosome pellet (SP<sub>1</sub> and SP<sub>2</sub>) and soluble (SS<sub>1</sub> and SS<sub>2</sub>) fractions were incubated in the presence of (<sup>3</sup>H) colchicine (final concentration 5 μM, specific activity 0.2 Ci/mM) for 90 min at 37°C in sodium phosphate buffer, pH 6.8 (see Chapter II, section 2.3.1) and bound colchicine was determined using the DE 81 filter disc method (see Chapter II, section 2.3.2).

### 6.2.3 Microtubule assembly

Microtubule assembly was investigated in both synaptosomal fractions (SS<sub>1</sub> and SS<sub>2</sub>) in the concentrated standard assembly buffer (see Chapter II section 2.2.1) containing 4M glycerol. For this purpose very small volumes of highly concentrated buffer solution were added in order to minimize dilution of the protein, since such dilution would reduce the chances for detecting assembly.

The possible formation of microtubules in SS<sub>1</sub> and SS<sub>2</sub> was examined by electron microscopy (see below) of samples that had been incubated at 37°C for 30 min in glycerol-assembly buffer.

### 6.2.4 Electron microscopy

Samples diluted with equal volume of assembly buffer were mounted onto grids and negatively stained with uranyl acetate as previously described (see Chapter II section 2.10). Photographs at different magnifications were taken from selected and representative areas of each grid.

### 6.3 Results

#### 6.3.1 Effects of microtubule stabilizing agents on the distribution of colchicine binding in synaptosomes

It can be seen from the data shown in table 6.1 and 6.2 that, in the absence of added stabilizing agents, the second synaptosomal fraction ( $SS_2$ ), which is thought to represent microtubular tubulin (i.e. derived from microtubules pelleted during the first centrifugation at room temperature and subsequently released in the cold), accounted for 13% to 15% of the total colchicine-binding activity in the lysed synaptosomes (i.e.  $SP_1 + SS_1$ ). Membrane-bound tubulin (in  $SP_2$ ), and non-polymerizable soluble tubulin (in  $SS_1$ ), each accounted for 40% to 45% of the remaining CB in the same fraction. This distribution is similar to that previously found when whole brains were extracted under similar conditions (see Chapter V, section 5.3.1).

Similarly, it is evident from the RSA values given in tables 6.1 and 6.2 that the solubilized microtubular fraction  $SS_2$  was considerably enriched in tubulin, suggesting that the cold-labile material released from the initial pellet prepared at room temperature ( $SP_1$ ) consisted, to a large extent, of a particulate form of microtubular protein (i.e. microtubules or other forms of cold-labile tubulin polymers) that is sedimentable with membranes during relatively short centrifugation at room temperature.

The inclusion of glycerol, and to a lesser extent of DMSO during the lysis step, selectively increased the proportion of CB activity that could be released by cold treatment in fraction  $SS_2$  from the initial pellet  $SP_1$ . Thus, it can be seen from the data shown in table 6.1 that twice as much activity was recovered in this 'microtubular' fraction when glycerol was employed, while an increase of 50% was seen with DMSO (Table 6.2). Moreover, these changes were accompanied by small increases in the protein recovered in fraction  $SS_2$ . However, when this is taken into account it can be seen that glycerol and to a lesser extent DMSO both induced an increase in the relative specific activity for colchicine binding in the  $SS_2$  fraction. Thus it can be concluded that the presence of glycerol, and to a lesser extent DMSO, induced a selective stabilization of microtubular structures.

It seems clear from the above data that a significant proportion of the tubulin (= CB activity) present in synaptosomes isolated by the Hajós procedure could be preserved as microtubular structures during lysis and extraction procedures that were developed to allow for the separation of microtubular tubulin from soluble and membrane-bound forms of the protein.

A key feature to the procedures was to take into account the known effects of temperature on microtubule integrity. It would be expected, therefore, that the yield of 'microtubular' tubulin (i.e. in particular, the CB- activity recovered in fraction  $SS_2$ ) should be significantly decreased if the lysis and subsequent extraction of the synaptosomes were carried out at  $4^{\circ}C$ .

For this purpose, further experiments were carried out : synaptosome preparations isolated and incubated in isotonic saline according to Hajós (see fig. 6.1 A) were divided into two equal portions, one of which was lysed and fractionated as described above (see also fig. 6.1 B) except that the temperature was maintained at 4°C throughout the lysis and fractionation steps.

Under these conditions , it is assumed that the most of the 'microtubular' tubulin would be extracted in the initial synaptosol fraction, SS<sub>1</sub>, instead of SS<sub>2</sub>, as is the case when the lysis and initial centrifugation steps were carried out at room temperature. To test this possibility further, experiments were also carried out in which the initial soluble (SS<sub>1</sub>) fraction prepared at 4°C or at RT<sup>o</sup> were incubated at 37°C in the presence of microtubule-assembly buffer containing 4M glycerol. Microtubular formation was assessed by electron microscopy (see fig. 6.8); CB-activity on any 'microtubule' pellet that might be formed was also measured.

It is evident from the data shown in table 6.3 that lysis and centrifugation of synaptosomes at 4°C resulted in a marked increase of CB-activity as in the initial soluble fraction SS<sub>1</sub> relative to that found in the SS<sub>2</sub> fraction. This indicates that part, at least, of the CB in SS<sub>2</sub> was derived from cold-labile microtubules. It is interesting to note, moreover, that even in the case of soluble extracts derived from synaptosomal pellets (SP<sub>1</sub>; see fig. 6.2) prepared at 4°C, SS<sub>2</sub> contained a considerable part of the total CB-activity recovered. Presumably



Table 6.1      Distribution of colchicine-binding activities in soluble and particulate subfractions of synaptosomes purified from chick brain and lysed, then incubated in the presence of glycerol.

Results shown are means of triplicate determinations from four separate experiments ( < 10% difference between experiments). Values in columns 2 and 4 are given as a percentage of binding activity or protein recovered from mother fractions: lysed synaptosomes and membrane + microtubular tubulin (SP<sub>1</sub>). S.A., specific activity, is expressed in c.p.m./mg protein. R.S.A, relative specific activity, = % bound c.p.m recovered/% protein recovered. Binding activities were assayed radiometrically as described in Chapter II, section 2.3.2. See also text, section 6.3.1.

Experimental conditions	Mother Fraction	Derived Fraction	Total bound cpm/fraction	Colchicine distribution %	Total protein mg/fraction	Protein distribution %	S.A	R.S.A
minus glycerol	Lysed synaptosomes	Pellet 1 (SP <sub>1</sub> )	325,000	61.0	12.2	61.0	26,600	1.0
		Synaptosol 1 (SS <sub>1</sub> ) containing (soluble tubulin)	207,000	39.0	7.8	39.0	26,500	1.0
minus glycerol	SP <sub>1</sub>	Pellet 2 (SP <sub>2</sub> )	234,500	75.5	9.9	92.5	23,700	0.8
		Synaptosol 2 (SS <sub>2</sub> ) containing (microtubular tubulin)	76,000	24.5	0.8	7.5	95,000	3.2
plus glycerol	Lysed synaptosomes	Pellet 1 (SP <sub>1</sub> )	398,500	67.5	14.0	69.0	28,500	0.98
		Synaptosol 1 (SS <sub>1</sub> ) containing (soluble tubulin)	191,800	32.5	6.3	31.0	30,500	1.05
plus glycerol	SP <sub>1</sub>	Pellet 2 (SP <sub>2</sub> )	207,800	51.5	11.1	89.5	18,700	0.57
		Synaptosol 2 (SS <sub>2</sub> ) containing (microtubular tubulin)	195,900	48.5	1.3	10.5	150,900	4.70

Table 6.1





Table 6.2      Distribution of colchicine-binding activities in soluble and particulate subfractions of synaptosomes purified from chick brain and lysed, then further incubated in the presence of

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DMSO.

Results shown are means of triplicate determinations from four separate experiments ( < 10% difference between experiments). Values in columns 2 and 4 are given as a percentage of binding activity or protein recovered from mother fractions: lysed synaptosomes and microtubular tubulin + membranes, (SP<sub>1</sub>). S.A, specific activity, is expressed in term of bound c.p.m recovered/mg protein recovered. R.S.A, relative specific activity, = % bound c.p.m recovered/% protein recovered. Binding activities were assayed radiometrically as described in Chapter II, section 2.3.2. See also text, section 6.3.1.

Experimental conditions	Mother Derived Fraction	Total bound cpm/fraction	Colchicine distribution %	Total protein mg/fraction	Protein distribution %	S.A	R.S.A
minus DMSO	lysed Synaptosome	Pellet 1 (SP <sub>1</sub> )	59.0	18.9	62.4	9,500	0.90
		Synaptosol 1 (SS <sub>1</sub> )	41.0	11.4	37.6	10,900	1.10
SP <sub>1</sub>	lysed Synaptosome	Pellet 2 (SP <sub>2</sub> )	78.0	16.9	93.0	8,800	0.80
		Synaptosol 2 (SS <sub>2</sub> )	22.0	1.3	7.0	32,500	3.10
plus DMSO	lysed Synaptosome	Pellet 1 (SP <sub>1</sub> )	59.4	20.1	66.0	11,800	0.90
		Synaptosol 1 (SS <sub>1</sub> )	40.6	10.4	34.0	15,600	1.20
SP <sub>1</sub>	lysed Synaptosome	Pellet 2 (SP <sub>2</sub> )	66.5	19.0	91.0	7,400	0.70
		Synaptosol 2 (SS <sub>2</sub> )	33.5	1.7	9.0	42,000	3.70

Table 6.2



Table 6.3     Distribution of colchicine-binding activities in soluble fractions of purified synaptosomes lysed at RT or 4°C in the presence of glycerol.

Results shown are means of triplicate determinations from two separate experiments. Values in column 2 are given as a percentage of binding activity recovered from the mother fraction SS<sub>1</sub>. S.A, specific activity, is expressed in term of bound c.p.m recovered/mg protein recovered.

See also text, section 6.3.1.

Experimental conditions	Fraction	Description	Total bound cpm/fraction	Bound cpm distribution %	Total protein mg/fraction	$10^3 \times$ S.A.
RT	Assembly ↙ SS1 MT3	{ Synaptosol 1 containing soluble tubulin	445,000	96.0	11.00	40.5
		{ Pellet 3 containing microtubules, MTs	16,000	4.0	0.65	25.5
	SS2	{ Synaptosol 2 containing MTlar tubulin	279,000		2.10	133.0
4°C	Assembly ↙ SS1 MT3	{ Synaptosol 1 containing soluble tubulin	696,000	96.0	13.0	53.5
		{ Pellet 3 containing microtubules, MTs	25,000	4.0	0.74	34.0
	SS2	{ Synaptosol 2 containing MTlar tubulin	113,000		1.65	68.5

Table 6.3

this is derived from a form of aggregated tubulin that was not releasable in the cold during the initial centrifugation of lysed synaptosomes (used to prepare  $SS_1$  and  $SP_1$ ), but become releasable subsequently during re-extraction of the  $SP_1$  pellet fraction used to prepare  $SS_2$ .

### 6.3.2 Microtubule assembly

Preliminary attempts to polymerize microtubules from  $SS_1$  in the presence of 4 M glycerol (at  $37^{\circ}\text{C}$ ) showed no more than the presence of very few ring structures (40-60 nm in diameter; see fig. 6.3). Attempts with  $SS_2$  preparations revealed the presence of scattered filaments (15 - 20 nm in width), generally short (ca. 1-2  $\mu\text{m}$ ), but occasionally much longer (see fig. 6.4). These filaments had a beaded appearance and were frequently ramified (see fig. 6.5 and 6.6) and in some areas, the branching seemed to originate from a central amorphous density, thus suggesting a possible site of nucleation (see fig. 6.6 and 6.7). Another interesting feature of the filaments polymerized from  $SS_2$  is their non-linear (denticulate) contour as compared to the usual smoother appearance of microtubules.

As discussed in the previous section (see also table 6.3).  $SS_1$  was enriched in CB-activity when the lysate was cooled at  $4^{\circ}\text{C}$ . Under the same condition, the CB-activity of  $SS_2$  (derived from  $SP_1$ ) decreased. This result is in a way, reflected by the negative results of attempts to detect polymerization in  $SS_2$  extracted under the same conditions, as compared to the

formation of filamentous structures observed when the same fraction ( $SS_2$ ) was extracted from a lysate treated at RT (cf. fig 6.4. and 6.8.). Only ring structures could be detected when  $SS_2$  was derived from cold-treated preparations (see fig. 6.8.).

#### Polymerization in the absence of glycerol

Attempts to polymerize microtubules from both  $SS_1$  and  $SS_2$  in the absence of glycerol were not successful. The only structures seen under the electron microscope, under such condition, were rings about 40 nm in diameter for both fractions (see fig. 6.9 and 6.10).

#### Effect of GTP

When the  $SS_2$  fraction was incubated in the presence of assembly buffer with no added glycerol, but with high concentrations of GTP (5-10 mM), few very short filaments could be observed under the electron microscope (see fig. 6.11). Branching was also noticeable, as well as some ring structures.

#### Effect of colchicine

When colchicine (50 M) was added to the  $SS_2$  fraction during incubation in the presence of glycerol, no polymerization was observed. Occasionally very small dense rings could be observed (see f.g. 6.12).





Fig 6.3 Random field of soluble tubulin-containing fraction or synaptosol 1 (SS<sub>1</sub>), incubated in microtubule assembly buffer in the presence of 4 M glycerol. Only ring structures (40-60 nm in diameter) were observed (see arrows).  
Magnification = 45,000.

Fig. 6.4 Representative filamentous structures seen in samples of microtubular tubulin-containing fraction, synaptosol 2 (SS<sub>2</sub>), incubated in microtubule assembly buffer in the presence of 4 M glycerol. Average length observed = 1 - 2  $\mu$ m, and width = 15 to 20 nm. See text, section 6.3.2, for details.  
Magnification = 72,000.

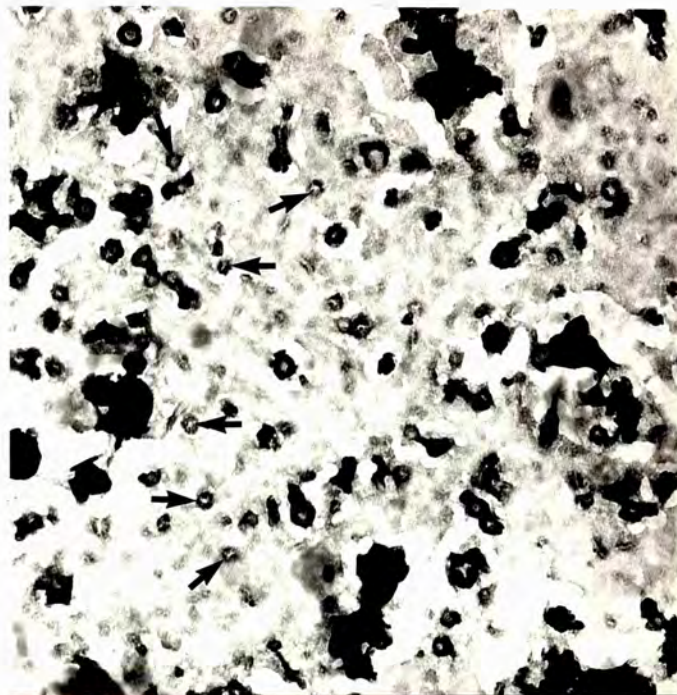


Fig. 6.3

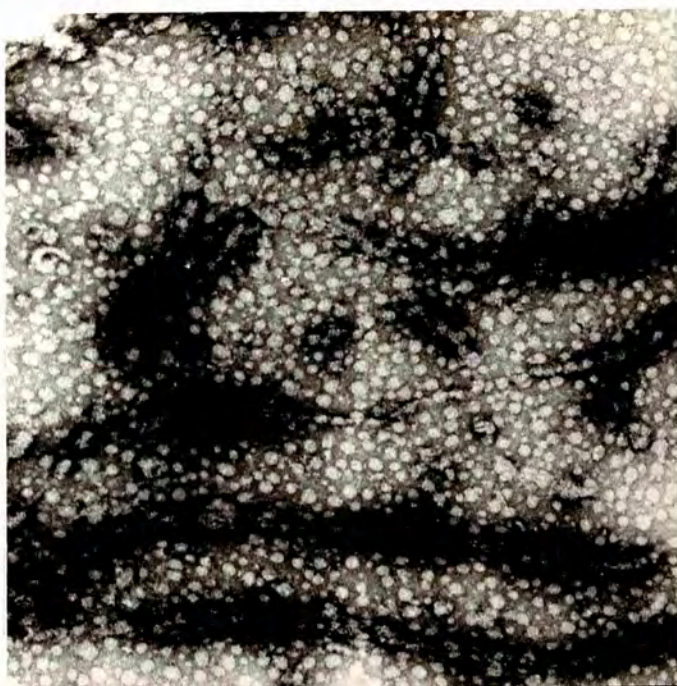


Fig. 6.4



Fig. 6.5 Representative filamentous structures seen in samples of incubated microtubular tubulin-containing fraction (see text, section 6.3.2), showing the beaded appearance referred to in text. Magnification = 45,000.

Fig. 6.6 Representative filaments polymerized from SS<sub>2</sub> fraction (see text, section 6.3.2 for details), showing the non-linear, denticulate, contour as compared to the usual smoother appearance of microtubules. Magnification = 72,000.

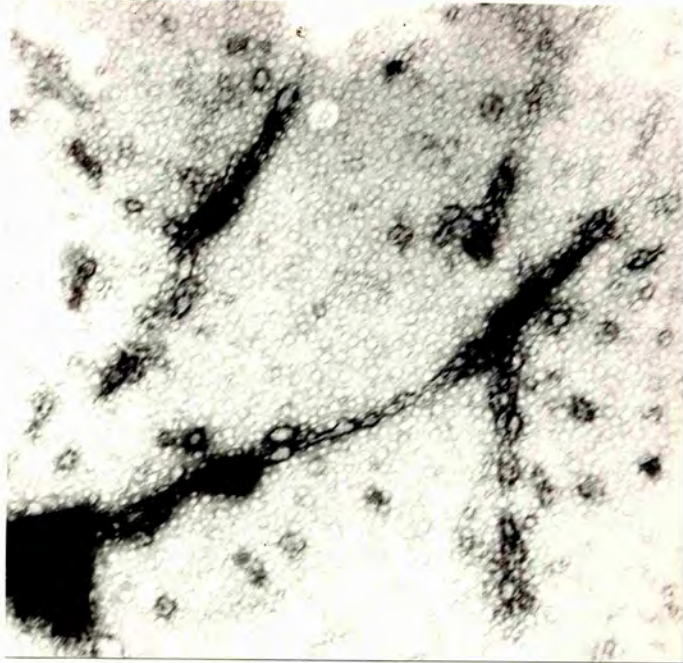


Fig. 6.5

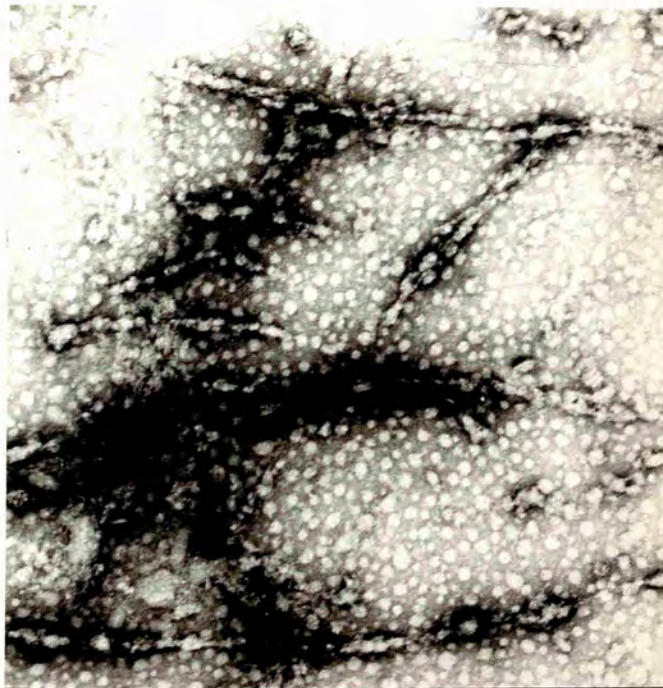


Fig. 6.6



Fig. 6.7 Representative filaments polymerized from SS<sub>2</sub> fraction (see text, section 6.3.2 for details), showing the branching of such structures from a central amorphous density (see arrows). Magnification = 45,000.

Fig. 6.8 Representative micrograph of microtubular tubulin-containing fraction, SS<sub>2</sub>, extracted from purified synaptosomes lysed in the cold. Incubation of SS<sub>2</sub> was carried out in assembly buffer in the presence of 4 M glycerol. Only ring structures (40-50 nm in diameter) could be observed under such conditions. See also text, section 6.3.2, and cf. fig. 6.4. Magnification = 45,000.





Fig. 6.7

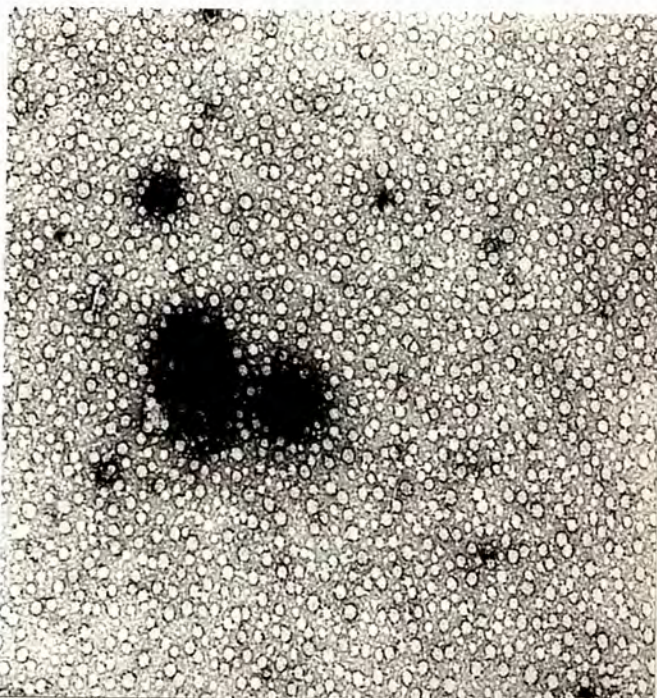


Fig. 6.8





Fig. 6.9 Representative micrograph of samples from the soluble tubulin-containing fraction, SS<sub>1</sub>, incubated in microtubule assembly buffer in the absence of glycerol. Under such conditions, only ring structures ( about 40 nm in diameter) could be observed. See also text, section 6.3.2 for details. Magnification = 36,000.

Fig. 6.10 Representative micrograph of samples from the microtubular tubulin-containing fraction, SS<sub>2</sub>, incubated in microtubule assembly buffer in the absence of glycerol. Under such conditions, only ring structures (about 40 nm in diameter) could be observed. See also text, section 6.3.2 for details. Magnification = 36,000.

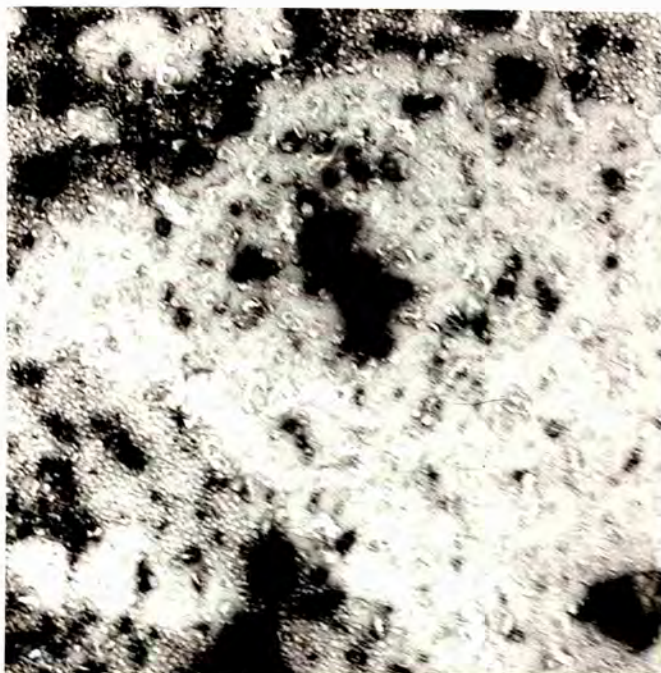


Fig. 6.9

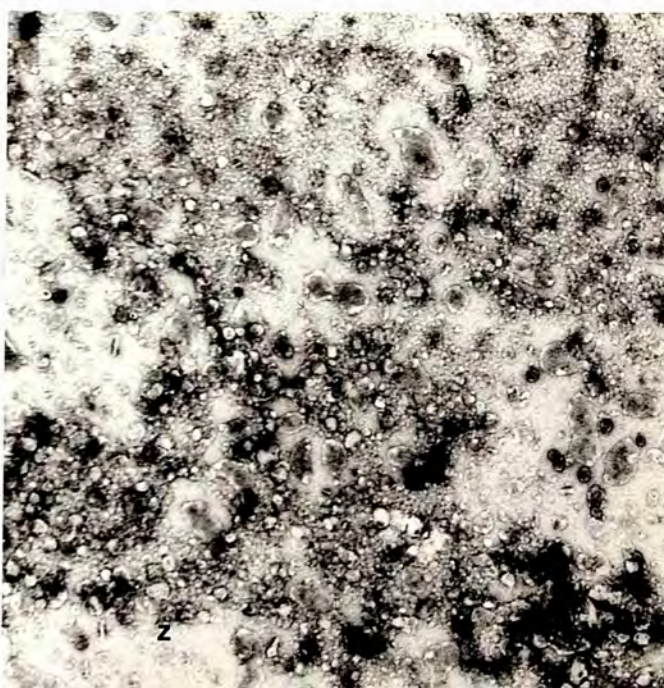


Fig. 6.10



Fig. 6.11 Representative micrograph of samples from SS<sub>2</sub> incubated in assembly buffer with no added glycerol, but with high concentrations of GTP. Under such conditions only very short filaments could be observed (their length not exceeding 1 micrometer). Note the branching appearance previously observed under glycerol incubation (cf. fig. 6.7). For more details, see text, section 6.3.2. Magnification = 14,500.

Fig. 6.12 Representative micrograph of samples from SS<sub>2</sub> incubated in assembly buffer in the presence of glycerol and 50  $\mu$ M colchicine. Under such conditions, only small dense ring structures were observed. See also text, section 6.3.2. Magnification = 11,000.



**Fjg. 6.11**



**Fjg. 6.12**



In conclusion, in no case were typical microtubular structures observed during assembly experiments using either fractions  $SS_1$  or  $SS_2$ . Only rings and filaments could be detected when the two fractions were incubated under the usual polymerization conditions. However, it is noteworthy that the filamentous forms observed were only detected when synaptosomes were prepared and extracted at **RT** in the presence of microtubule stabilizing agents, and in those subfractions exhibiting high colchicine-binding activity. This indicates that the polymers formed were partly, if not completely derived from, or dependent on the presence of microtubular proteins.

### 6.3.3 Gel electrophoresis

Samples from  $SS_1$ ,  $SP_1$ ,  $SS_2$  and  $SP_2$  were run on SDS-PAGE in the presence of urea. The pattern of proteins obtained in each case is described below. In these experiments purified tubulin contaminated with protein of MW 45,000 was used as a reference marker.

#### Fraction $SS_1$

The relatively low concentration of protein in this fraction did not allow a loading of more than  $20\mu\text{g}$  of sample on the gels. Nevertheless, this showed clearly the presence of a double band corresponding to  $\alpha$  and  $\beta$  tubulin (MW 54 000 and 57 000) on the basis of its comigration with purified tubulin added as a marker (see fig. 6.13). The gel also showed the presence

of a band (in  $SS_1$ ) comigrating with the contaminant band (MW 45 000) of the purified tubulin. It is perhaps noteworthy that the band corresponding to  $\alpha$  tubulin was more intense than that comigrating with the  $\beta$  tubulin of the marker.

However, subsequent incubation of  $SS_1$  under microtubule assembly conditions (in assembly buffer containing 4 M glycerol) did not give rise to any filamentous structures when samples were examined under the electron microscope (see section 6.3.1). Nonetheless, a minute pellet could be collected when the incubated solution was centrifuged at 100,000 g for 60 min. at room temperature (see fig. 6.2). Electrophoresis of this pellet yielded two discrete bands which comigrated, respectively, with the  $\alpha$  tubulin and with the 45,000 MW contaminant bands of purified tubulin (Fig 6.13). The apparent absence of the  $\beta$  comigrating band in the pellet fraction under these conditions suggests that the sedimented material did not represent a typical polymerization product of tubulin and indeed no filament structures were observed during the assembly incubation when samples were examined by electron microscopy. Furthermore, it would also appear that the tubulin-like proteins seen in unincubated samples of  $SS_1$  may represent forms of tubulin which were not polymerization-competent even though they comigrated with  $\alpha$  and  $\beta$  components of purified tubulin.

#### Fraction $SP_1$

In this fraction, the pattern of protein shows three distinct bands comigrating with the  $\alpha$ ,  $\beta$  and the 45 000 MW contaminant bands of the purified tubulin marker. However it should be noted



that the  $\alpha$ - and  $\beta$ -like bands of this fraction are separated by a gap wider than that observed in the double band from purified tubulin. Furthermore, the  $\alpha$ -like band seems to correspond more precisely to the slower migrating portion of the  $\alpha$  tubulin band, thus suggesting heterogeneity amongst the tubulin-like protein (e.g.  $\alpha_1, \alpha_2$ : see Chapter I) in the present preparation, the slower component ( $\alpha_1$ ) being preferentially found in  $SP_1$  (see fig. 6.14). In addition, it can be seen that a number of bands (indicated by small arrows, Fig 6.14) are characteristically seen in  $SP_1$ . The intense protein staining seen in the region of the dye-front, probably represents protein degradation products as well as native small MW polypeptides.

#### Fraction $SS_2$

In this fraction as well the band comigrating with  $\alpha$  tubulin seems to correspond mainly to the slower-migrating portion of the marker protein and represents, as judged by the dye intensity, one of the two major proteins present in  $SS_2$  (fig 6.15). The other major protein seen as migrated ahead of the 45 000 MW contaminant of the marker tubulin. Such a band was not obvious in the previous fractions (cf  $SS_1$  and  $SP_1$ , Figs 6.13 and 6.14). Once again, intensely staining material was accumulated in the region of the dye-front. Finally, it is evident that relatively little material corresponding to  $\beta$  tubulin was apparent in fraction  $SS_2$ .

#### Fraction $SP_2$

The pattern of proteins seen for  $SP_2$ , was similar to that already observed for the other particulate fraction  $SP_1$ .



Fig. 6.13 Gel electrophoresis of the soluble tubulin fraction, SS<sub>1</sub>, and of assembly material derived from it, MT<sub>3</sub>.

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Samples from SS<sub>1</sub> fraction and MT<sub>3</sub> pellet, were run on SDS-PAGE in the presence of urea. Electrophoresis was carried out in tris-glycine buffer systems. In the case of SS<sub>1</sub>, the gel shows clearly the presence of a double band comigrating with the  $\alpha$  and  $\beta$  tubulin of purified tubulin added as a marker (54,000 and 57,000 for  $\alpha$  and  $\beta$  respectively), whereas MT<sub>3</sub> gels showed the presence of an comigrating band only. In both fractions a band comigrating with the contaminant band (MW 45,000) of the purified tubulin (see text, section 6.3.3).

Fig. 6.14 Gel electrophoresis of the 'microtubular tubulin + membranes' fraction, SP<sub>1</sub>.

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Samples from SP<sub>1</sub> fraction were run on SDS-PAGE in the presence of urea as described above. Three distinct bands comigrating with the  $\alpha$ ,  $\beta$  and the 45,000 MW contaminant bands of the purified tubulin marker could be observed. Note that the  $\alpha$ -like band seems to correspond to the slower migrating portion of tubulin or  $\alpha_1$  high molecular weight (HMW), as well as intermediate molecular weight (IMW), protein are also observed. Arrows refer to small molecular weight protein characteristic to SP<sub>1</sub> (not observed in soluble fractions, SS<sub>1</sub> and SS<sub>2</sub>). Finally note the intense staining near the dye front, Df. See also text, section 6.3.3.

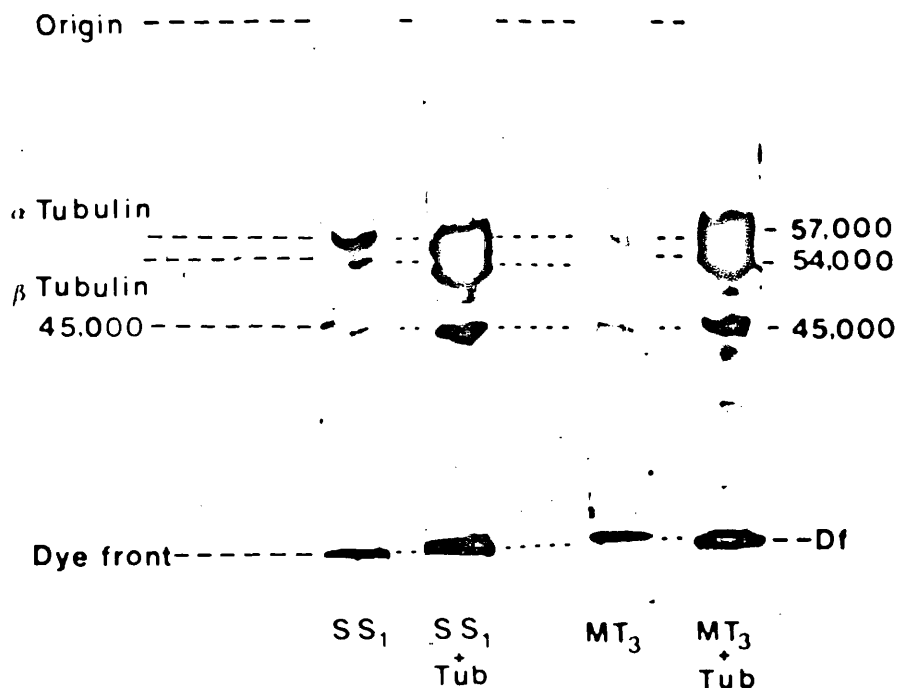


Fig. 6.13

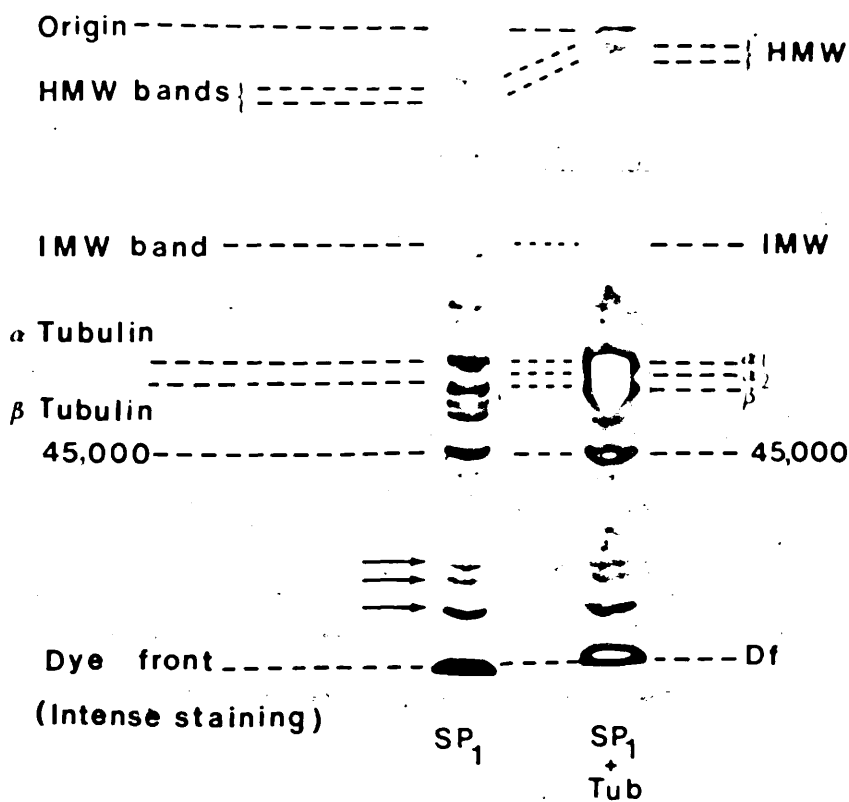


Fig. 6.14



Fig. 6.15      Gel electrophoresis of the microtubular tubulin fraction, SS<sub>2</sub>.

Samples from SS<sub>2</sub> fraction were run on SDS-PAGE as previously described (see figs. 6.13 & 6.14). The gel shows the presence of two major bands: one corresponding to the slower-migrating portion of the  $\alpha$  tubulin marker, the other migrating ahead of the 45,000 MW contaminant of the marker protein (Fast-travelling component, FC). The FC band was not obvious in the previous fractions (i.e. SS<sub>1</sub> & SP<sub>1</sub>). Note the relatively little material corresponding to  $\beta$  tubulin and the intensely stained material near the dye front. See also text, section 6.3.3.

Fig. 6.16      Gel electrophoresis of the membranes fraction, SP<sub>2</sub>.

Samples from SP<sub>2</sub> fraction were run on SDS-PAGE as described previously (see figs. 6.13 & 6.14). The pattern of protein on gel is similar to that observed for SP<sub>1</sub> except for the fast-travelling band, FC (see above), which was obvious in this case, though less prominent than that found in SS<sub>2</sub>. More details in text, section 6.3.3.

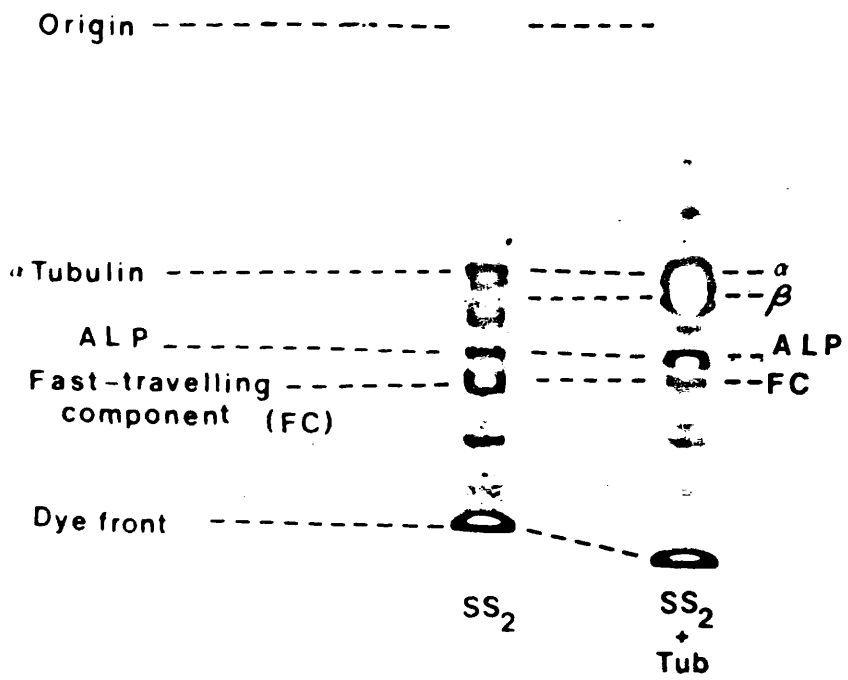


Fig. 6.15

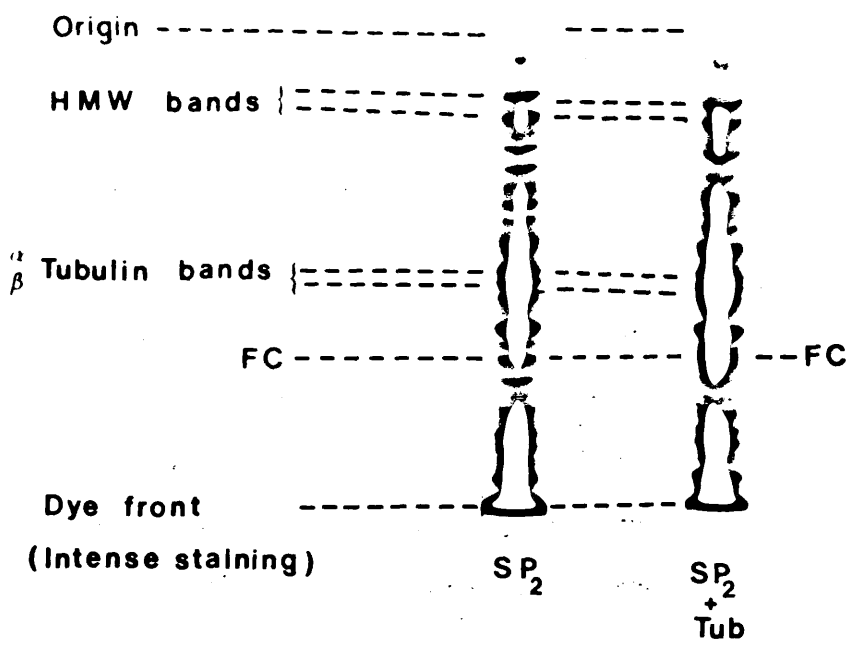


Fig. 6.16

However, it should be noted that an additional band, migrating ahead of the 45 000 MW contaminant (see arrow fig 6.16) was also observed, similar though less prominent than that found in  $SS_2$ , the other subfraction derived from  $SP_1$  (cf Fig 6.15). This band was barely detectable in the initial subfraction of the lysed synaptosome preparation,  $SP_1$  and  $SS_1$ , though as noted earlier, it represented a major component of the so-called 'microtubular' fraction,  $SS_2$ . Since the relative increase of this component in  $SS_2$  seemed to coincide with the low content of  $\beta$ tubulin comigrating material in this fraction, it is conceivable that this faster-migrating band represents a degradation product of tubulin (see Discussion).

#### 6.4      Conclusions

The following conclusions may be drawn from the foregoing experiments :

1 - Stabilizing agents and higher temperatures promote the formation and/or stabilization of a CB fraction which can be sedimented from lysed synaptosome preparations together with membranes ( $SP_1$ ), and subsequently released from this fraction under conditions favouring microtubule disassembly (i.e. in the cold) to yield a fraction,  $SS_2$ , that was highly enriched in CB activity.

On this basis it is tentatively concluded that fraction  $SS_2$  was enriched in tubulin subunits that were derived from polymerized microtubular structures that were formed and preserved when synaptosomes were prepared according to the method of Hajós and fractionated in the presence of microtubule stabilizing agents.



The sedimentation properties of CB proteins under these conditions are similar to those found when using microtubule stabilizing agents to prepare crude soluble and particulate fractions from whole brain. It was also noted, during the present experiments, that some form of aggregatable CB protein behaving similarly to microtubular protein was also present, though to a much smaller extent, in the initial synaptosomal pellet,  $SP_1$ , from which it could be subsequently released in the cold, in  $SS_2$ , when synaptosomes were initially prepared and incubated under conditions not favouring microtubule assembly (see table 6.3 and controls in table 6.1 and 6.2). In these control experiments there was a concomitant increase in the proportion of CB protein i.e. tubulin recovered in the initial soluble fraction  $SS_1$  (see tables 6.1 - 6.3).

Thus under conditions favouring the assembly and preservation of microtubules, fraction  $SS_1$  would mainly represent soluble non-microtubular and non polymerizable form of tubulin, and this fraction of tubulin was indeed increased as judged by CB data in control experiments (see tables 6.1 - 6.3).

A true assessment of the microtubular tubulin present in fraction  $SS_2$  should take into account the contribution of the 'background' CB activity released in fraction  $SS_2$  when this was derived from synaptosomes prepared under control conditions.

2 - The observations that only fraction  $SS_2$  prepared from synaptosomes incubated in the presence of stabilizing agents, yield filamentous structures, after incubation under microtubule assembly conditions (fig 6.4), are in keeping with the

conclusion that, part at least, of the CB activity present in fraction  $SS_2$  represents microtubule-derived polymerization-competent tubulin. However, it is evident that the filaments seen did not give the appearance of typical microtubule structure. Since such filaments were not seen in the presence of colchicine (fig 6.12) or in the cold (fig 6.8) it seems reasonable to assume that those filaments do indeed represent a typical tubulin polymer. The reasons of this are not clear. However, it is interesting that the main protein seen after electrophoresis of fraction  $SS_2$ , as compared to that seen for fraction  $SS_1$  (cf fig 6.15 and 6.13 respectively), is represented by a faster migrating band (MW less than 45 000, see fig 6.15 and accompanying text) and that the and in particular the comigrating peptides were present in relatively low concentrations. Thus the so-called microtubular fraction that was enriched in CB activity was not apparently enriched in the typical  $\alpha$  and  $\beta$  components of tubulin. One possibility which would require further investigation is that the prominent fast migrating component, together possibly with material accumulated at the dye-front represent degradation products of tubulin which have retained the CB activity but lost the ability to form typical microtubule polymers (see Chapter VII for further discussion).

Furthermore it is notable that fraction  $SS_2$  did not appear to contain detectable amounts of the minor high molecular weight (HMW) components of MW ca 300,000 - 350,000, typically

found in polymerization-competent preparations of microtubular protein (see Chapter I section 1.32). It should be stressed however, that the apparent absence of these minor HMW proteins could be due either to their degradation (see Chapter VII) or to the lack of sensitivity in the experimental methods employed.

Chapter VII

Discussion and Conclusions

## Chapter VII    Discussion and Conclusions

The main aim of the studies reported in this thesis was to investigate the polymerization-competence of tubulin present in nerve endings. Interest in this area stems from the recent discovery, that microtubules can be visualized in the presynaptic regions of axons in situ using special procedures of fixation and staining (Gray, 1975,1976).

Earlier studies had shown that tubulin was present in soluble extracts of synpatosomes prepared from rat brain as well as membrane associated subfractions of rat brain synaptosomes (Lagnado et al 1971; 1975).

Chick brain was early shown to be a rich source of micro-tubular protein, but had not been previously employed to investigate its subcellular distribution. It was important first to establish a subcellular fractionation method suitable to chick brain, since until recently no such method was available.

A first approach involved the application of the method of Jones and Matus (see Chapter III), which was adapted to rapidly separate synaptosomal soluble and membrane subfractions, in which the distribution of colchicine binding could be studied. Using this method, it was shown that both the soluble and SPM-containing fractions were enriched in CB activity. During this work it was found that a membrane fraction enriched in myelin also bound colchicine, but the CB in this fraction was atypical, since it was not stabilized by vinblastine. A similar observation was previously reported for microsomal membranes

derived from secretory tissues (e.g. pancreas) by Redman et al (1975). Furthermore, it was found during investigations of subcellular distribution of vinblastine-binding activity, that myelin showed the highest binding activity for this alkaloid since it is known that vinblastine may interact with components other than tubulin (Wilson et al 1970), these results emphasise that some caution is necessary in interpreting the use of CB data as sole indication of the presence of microtubular protein. This is in keeping with the view that the observed effects of antimitotic alkaloids on neural function may not exclusively result from their direct interaction with microtubular subunit proteins (Itani & Lagnado, 1976).

In the course of this work, an adaptation of the Jones and Matus method, specifically designed to obtain synaptosomal subfractions from chick brain (Leeuwan et al, 1976) appeared in the literature. This method took into account minor differences observed in the sedimentation properties of chick brain membrane components, as et al. compared to those of rat brain (see Leeuwan et al, 1976 and Babitch 1976). In their work Leeuwan et al did not in fact carry out any biochemical investigations on the 'synaptosol' fraction. The results described in Chapter IV clearly indicate that the content of CB activity in the synaptosol fraction prepared by the Leeuwan method was twice as high as that found in the synaptosomal fraction prepared by the original Jones and Matus method. This difference could be due to a better preservation of tubulin (in terms of its CB activity) in the synaptosome fraction, and/or to a higher yield of synaptosomes in the crude mitochondrial fraction from which synaptosomal subfractions were derived by the method of Leeuwan et al.

For the purposes of investigating the properties of synaptosomal tubulin, the method of Leeuwen *et al* is thus clearly advantageous, and it was used in further work to study the polymerization competence and colchicine binding of synaptosomal tubulin (see Chapter IV).

However, preliminary investigations failed to demonstrate the polymerization of tubulin in chick brain synaptosomal preparations incubated under typical microtubule assembly conditions. Possible reasons for these negative results were discussed earlier (see Chapter V) and led to preliminary studies of the effects of microtubule stabilizing agents and temperature on the preservation of microtubular structures in crude soluble and particulate preparations from rat brain homogenates.

From further work described in Chapter V, based on the use of colchicine-binding assays, it is apparent that microtubule derived tubulin represented only 15% of the total tubulin present in stabilized homogenates, as compared to the soluble, non-polymerizable fraction of tubulin which accounted for 40-45% of the total homogenate tubulin. When these data are taken into account, it is evident that only a small proportion of the total tubulin present in synaptosomes could be considered as polymerisation-competent tubulin (see below), assuming that a similar proportion of the synaptosome soluble tubulin was derived from microtubular structures present in isolated synaptosomes.

The results described in Chapters IV and VI show clearly that around 10% of the total tubulin in synaptosomes were extracted into the soluble fraction, assuming that only about 15% of this material represented polymerization-competent tubulin (see above), the amount of synaptosomal tubulin that could be expected to polymerize

represents, in terms of CB activity, less than about 2% of the total. This clearly represents a very low proportion of the total tubulin present in nerve endings, which may partly explain the complete failure (see Chapter IV) or limited success (see Chapter VI) in obtaining any microtubules from synaptosols incubated under assembly conditions.

Another reason why no microtubules could be polymerized from soluble synaptosomal extracts may be the lack of microtubule-associated proteins. To test this possibility, small amounts of concentrated high-speed supernatant from chick brain, which contained assembly-competent tubulin, were added to the synaptosol during the assembly incubation. Such material could provide sufficient microtubule-associated protein to stimulate microtubule assembly (see Chapter IV). Under these conditions, still no assembly was noted, which suggests that one could be dealing with a totally different form of tubulin, or tubulin-like proteins, that bind colchicine, but are not readily polymerizable under typical microtubule assembly conditions. Indeed, while gel electrophoresis of the synaptosol fraction prepared using two different methods (Leeuwman et al 1976 and Hajos 1975) showed the presence of both  $\alpha$ - and  $\beta$ -like protein bands, the  $\alpha$ -like band was atypical in that it comigrated with the slower component of  $\alpha$  tubulin, which was referred to as ' $\alpha_1$ ', (see Chapters IV and VI).

Furthermore, it was found that synaptosol fractions enriched in 'microtubular' tubulin (see Chapters VI), contained as a major additional component a faster-migrating protein. The nature of this fast migrating component is not known. However, it is tempting to speculate that since tubulin was clearly present as a major component in the synaptosol fraction, originally prepared without stabilizing agents, the appearance of this additional component in soluble fractions of



synaptosomes extracted in the presence of stabilizing agents at room temperature, and the relative absence of typical tubulin components in this fraction, results from the degradation of tubulin during the preparation of SS under these conditions. In this view<sup>2</sup> glycerol and room temperature treatments can be conceived as preserving the activity of soluble proteases acting on tubulin, a possibility that is well worth further study in view of the well known enrichment of the synaptosomes in proteolytic enzymes active under the pH and ionic conditions used in this work (see Guroff, 1964). Thus a more successful approach to study the assembly competence of synaptosomal tubulin might be to carry out the experiments in the cold, in the absence of stabilizing agents, but in the presence of proteases inhibitors.

A most interesting observation from this work is that despite the apparent limited degradation of tubulin discussed above, CB activity was clearly preserved and indeed, on the basis of CB assays, it was possible to define a microtubular tubulin fraction despite the inability of this fraction to form typical microtubular structures in vitro under the usual assembly conditions. This indicates that the structural requirements of tubulin for colchicine binding and for assembly may be quite different. A similar difference was recently noted in studies where the effects of SH reagents on CB activity and polymerization were compared (Kuriyama and Sakai, 1974 and Mellon and Rebhun 1976).

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Seven experimental groups each composed of 1N, 1L and 1D animal were used, together with a further group containing 2N animals and 1D animal. Mean specific radioactivities for each fraction were calculated for each experimental group and normalized around a mean specific radioactivity in the homogenate of 1000 d.p.m./mg of protein. In addition the specific radioactivity ratios of L/N, D/N and L/D were calculated for each fraction in each experimental group. The normalized specific radioactivities are given in Table 1. There were no significant differences, or trends approaching significance, for any fraction or condition in the motor cortex. All observed effects thus showed a regional specificity. The  $43 \pm 17\%$  greater homogenate specific radioactivity for L rats as compared with D rats essentially replicates our earlier observations (Richardson & Rose, 1972). In the  $P_1$  fraction (for nomenclature, see Table 1), the value for L rats was higher, and for D rats lower, than that for N rats, and the significant difference was a  $50 \pm 22\%$  greater incorporation in L rats as compared with D rats. This too is compatible with our earlier observation that there is enhanced incorporation into a ribosomally bound fraction in the visual cortex of L animals (Jones-Lecointe *et al.*, 1976), as under these conditions of homogenization and centrifugation, ribosomes will appear in the  $P_1$  fraction. There were no significant differences in specific radioactivity between conditions in the  $S_2$  fraction (nor in the  $S_1$  fraction from which  $S_2$  and  $P_2$  are derived). However, comparing incorporation in the  $P_2$  polymerized tubulin fraction, incorporation was  $64 \pm 19\%$  greater in N rats, and  $110 \pm 28\%$  greater in L rats, as compared with D rats. This difference is the largest that we have found in any protein fraction in our experiments to date.

We conclude that incorporation of precursor into, and hence probably the synthesis of, a tubulin-enriched fraction is decreased in the visual cortex of dark-reared rats. On exposure to the light, there is a substantial increase in the synthesis of  $P_2$  protein, resulting in a doubling of the incorporation rate in the first hour of light exposure. Whereas the  $P_2$  fraction contains minor components other than tubulin, it would seem unlikely that a change of this magnitude in the specific radioactivity of the whole fraction could occur as a result simply of a change in such minor components. This would require a manyfold increase in their specific radioactivity, and comparable increases have not been observed in minor components of the soluble cell cytoplasm separated by chromatographic techniques.

Fellous *et al.* (1975) have claimed that amounts of tubulin in brain vary markedly during early development in the rat, whereas Cronly-Dillon & Perry (1975) reported that both the amount and rate of synthesis of tubulin increase substantially in the visual cortex in the period around eye opening in the rat. However, it is not clear from such observations if this increase is dependent on functional stimulation. The present experiments suggest that the rate of tubulin production in the visual cortex is indeed related to functional stimulation, and increases rapidly on the onset of such stimulation.

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### The Distribution of Colchicine and Vinblastine Receptors in Subcellular Fractions from 1–3-day-old Chick Brain

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Colchicine and the *Vinca* alkaloids, vinblastine and vincristine, are commonly used as probes for microtubule-dependent processes in various cell types (see, for example, Oliver *et al.*, 1974; Edelman *et al.*, 1974; Nicolson, 1976; McClure, 1972): both drugs are known to inhibit selectively microtubule assembly *in vitro* and *in vivo* and to interact, through separate high-affinity binding sites, with tubulin, the subunit protein of microtubules (Wilson *et al.*, 1975; Olmsted & Borisy, 1973). Further, colchicine-binding studies in subcellular fractions from mammalian brain provide indirect evidence that tubulin is firmly associated with isolated synaptic junctional complexes, as well as being present in perikaryal and synaptosomal soluble fractions (for references, see Lagnado *et al.*, 1975). This was corroborated by more direct evidence based on chemical analysis of purified synaptic-junctional fractions enriched in post-synaptic density material (Walters & Matus, 1975) and by immunohistochemical (Matus *et al.*, 1975) and electron-microscopic observations (Gray, 1975) on cerebral-cortex tissue *in situ*.

Taken together, these observations suggest that some of the observed pharmacological effects of anti-tubulin drugs on synaptic function (see, e.g., Katz, 1972; Hanbauer *et al.*, 1974; Wooten *et al.*, 1975; Poisner, 1973; Sorimachi *et al.*, 1973) may be related to a direct interaction of the drugs with tubulin associated with synaptic components. To investigate further this possibility, the distribution of colchicine- and vinblastine-binding sites in subcellular fractions from chick brain was studied, with particular reference to subfractions derived from synaptosomes.

Whole brain from 1–3-day-old chicks was homogenized and fractionated by the method of Jones & Matus (1974), except that 2mM-sodium phosphate buffer, pH 6.8, and 50  $\mu$ M-MgCl<sub>2</sub> was incorporated into the sucrose solutions used throughout. The crude mitochondrial pellet (P<sub>2</sub>) was lysed for 30 min at 4°C in 2mM-sodium phosphate buffer, pH 8.1, and spun at 100000g<sub>av.</sub> for 30 min to extract the synaptosol (P<sub>2</sub>-sol), before further subfractionation of the particulate material. The crude post-mitochondrial supernatant (S<sub>2</sub>) was spun at 100000g<sub>av.</sub> for 60 min to yield a crude microsomal (P<sub>3</sub>) and soluble (S<sub>3</sub>) fraction. Colchicine-binding activity was assayed in freshly obtained fractions as previously described (Lagnado *et al.*, 1971), except that vinblastine (100  $\mu$ M, final concn.) was incorporated in the assay mixtures which contained 200–400  $\mu$ g of protein in a final volume of 0.5 ml. Vinblastine binding was assayed in a similar manner (Owells *et al.*, 1974), by using 5  $\mu$ M-[G-<sup>3</sup>H]vinblastine sulphate (sp. radioactivity, 30 Ci/mol; The Radiochemical Centre, Amersham, Bucks., U.K.; batch 1).

The results summarized in Table 1 show that the distribution of binding activities for both drugs in subfractions of the postnuclear (S<sub>2</sub>, P<sub>2</sub>) and postmitochondrial (S<sub>3</sub>, P<sub>3</sub>) supernatants (S<sub>1</sub> and S<sub>2</sub>, respectively) were very similar and that for each drug, only a slight enrichment in binding activity was seen in the respective soluble fractions obtained (Table 1, last two columns). By contrast, marked differences were observed in the relative distributions of binding activities among the subfractions derived from the lysed crude mitochondrial fraction. The distribution of colchicine-binding sites was similar to that found previously for mammalian brain (Lagnado *et al.*, 1971), showing a relative enrichment in the synaptosol and synaptic-membrane-enriched fractions, but relatively little activity in other fractions. Vinblastine-binding sites, on the other hand, were concentrated primarily in the myelin fraction, which also contains light membranes, whose origin has not been determined. It may be noteworthy, in this connection, that although vinblastine stabilized colchicine-binding activity in the synaptosol and synaptic-plasma-membrane fractions, colchicine binding in the myelin fraction was consistently inhibited by about 20% in the presence of 100  $\mu$ M-vinblastine (A. N. Itani, unpublished work). In addition, the fraction containing synaptic plasma membranes was

Table 1. *Distribution of colchicine(C)- and vinblastine(Vb)-binding activities in subfractions of chick brain postnuclear supernatant*

Results shown are means of triplicate determinations from three separate experiments (<10% difference between experiments). Values in the first three columns are given as a percentage of protein or binding activity recovered from mother fractions: (S<sub>1</sub>), postnuclear supernatant; (S<sub>2</sub>), post-mitochondrial supernatant; (P<sub>2</sub>), lysed crude mitochondrial preparation. Binding activities were assayed radiometrically as described in the text.

Fraction	Description	Protein (%)	Binding activity				Relative sp. radioactivity (% bound c.p.m. recovered / % protein recovered)	
			Distribution (%)		10 <sup>-3</sup> × Sp. radioactivity (bound c.p.m./mg of protein)			
(S <sub>1</sub> ) S <sub>2</sub>			C	Vb	C	Vb	C	Vb
	Postmitochondrial supernatant	79	81	82	29.6	30.6	1.02	1.04
P <sub>2</sub>	Crude mitochondria (lysed)	21	19	18	28.4	26.7	0.91	0.86
(S <sub>2</sub> ) S <sub>3</sub>	Postmicrosomal supernatant	43	54	48	43.1	36.8	1.26	1.12
P <sub>3</sub>	Crude microsomal fraction, small nerve endings	57	46	52	27.4	29.8	0.81	0.91
(P <sub>2</sub> ) P <sub>2</sub> -sol	Synaptosol	23	33	25	56.5	49.0	1.43	1.09
My	Myelin light membranes	5	4	14	24.4	93.3	0.80	2.80
SPM	Synaptic plasma membranes	19	23	26	50.4	52.8	1.22	1.36
Mit	Mitochondria	53	40	35	32.2	24.5	0.76	0.66

also enriched in vinblastine-binding activity, whereas little enrichment was seen in the synaptosomal fraction as compared with that found for colchicine binding.

Further work is clearly required to establish whether the differences observed in the distribution of colchicine- and vinblastine-binding activities among subsynaptosomal components are related to intrinsic differences in the nature of the receptors involved, or to other factors which might differentially affect the binding reactions for the two drugs. The possibility that *Vinca* alkaloids can also interact with non-tubulin components in other tissues has already been noted (see, e.g., Wilson *et al.*, 1970). In conclusion, it is evident that some caution is needed in interpreting the observed effects of antimitotic alkaloids on neural function as resulting initially, from a direct interaction with microtubular subunit proteins.

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### The Incorporation *in vivo* of [<sup>32</sup>P]Orthophosphate into Phospholipids Associated with Brain Microtubular Proteins

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Microtubules purified from 1–3-day-old chick brain by two cycles of reassembly *in vitro* contain 2–4 μg of phospholipid P per mg of protein. The specific radioactivity of microtubule-associated phospholipids from chick brain labelled for 2 h after intracerebral injection of [<sup>32</sup>P]P<sub>i</sub> (200–400 μCi/animal) was similar to that found as phosphoprotein P (Lagnado *et al.*, 1975; Lagnado & Kirazov, 1975), the greatest amount of labelling occurring in the phosphoinositide fraction.

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