

THE EFFECTS OF INDOLE ALKALOIDS AND RELATED COMPOUNDS  
ON THE PROPERTIES OF BRAIN MICROTUBULAR PROTEINS



THE EFFECTS OF INDOLE ALKALOIDS AND RELATED COMPOUNDS  
ON THE PROPERTIES OF BRAIN MICROTUBULAR PROTEINS

A thesis submitted

by

TAN LUCK PHENG

to the

Faculty of Science

for the

Degree of Doctor of Philosophy

in the

University of London

Department of Biochemistry,  
Bedford College,  
Regent's Park,  
London, NW1.

February, 1975.

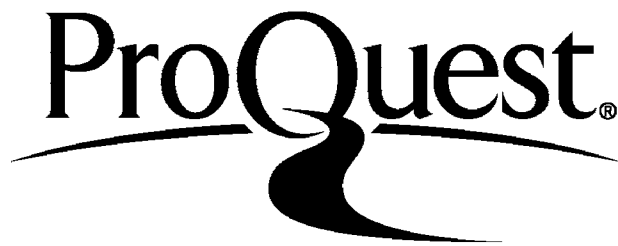
ProQuest Number: 10098287

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



ProQuest 10098287

Published by ProQuest LLC(2016). Copyright of the Dissertation is held by the Author.

All rights reserved.

This work is protected against unauthorized copying under Title 17, United States Code.  
Microform Edition © ProQuest LLC.

ProQuest LLC  
789 East Eisenhower Parkway  
P.O. Box 1346  
Ann Arbor, MI 48106-1346

ABSTRACTTHE EFFECTS OF INDOLE ALKALOIDS AND RELATED COMPOUNDS ON THE  
PROPERTIES OF BRAIN MICROTUBULAR PROTEINS.

Microtubular subunit protein (tubulin) was isolated from foetal rabbit brain extracts by chromatography on DEAE-Sephadex or by in vitro polymerisation procedures. The effects of indole alkaloids and related compounds on the colchicine and nucleotide binding properties of tubulin were investigated using sensitive radiochemical assay procedures developed for this purpose. It was shown that in contrast to the stabilising effects of Vinca alkaloids (vincristine and vinblastine), a number of structurally related drugs containing methoxyindole derivatives (e.g. reserpine, melatonin, harmine) inhibited the binding of colchicine to the protein. However, both groups of indole alkaloids markedly enhanced the binding of guanosine nucleotides to tubulin. During the course of this work, the specificity of nucleotide binding sites present in isolated microtubular protein preparations was also investigated.

The effects of indole derivatives on the in vitro assembly of brain microtubules were studied by electron microscopy: various Vinca alkaloids as well as colchicine caused either partial or complete block of microtubule assembly, while melatonin promoted the appearance of 'twisted' microtubules. However, the reassembly of microtubules in the presence of reserpine appeared to be normal. Additional experiments are reported concerning the binding of [<sup>3</sup>H] vincristine to isolated tubulin and the subcellular distribution of vincristine binding receptors in brain homogenates. A short collaborative study on the phosphorylation of microtubular proteins in vitro and in situ is also reported.

ACKNOWLEDGEMENTS

I thank Professor D.F. Cheesman for giving me an opportunity to work in his Department and for his advice. I am indebted to my supervisor, Dr. J.R. Lagnado, for his continued interest, guidance and encouragement throughout the course of this work.

I am grateful to my colleagues in the "Microtubule Lab" for their help and psychological support, in particular to Dr. M. Reddington with whom I have shared many helpful discussions and to Mrs. C.A. Lyons who kindly introduced me to a number of techniques employed in the present work. My thanks are also due to all the technical staff in the Department, especially to Mr. E.C. Hawkes, F.I.S.T. who was responsible for producing the final Figures presented in this thesis. I thank Mr. R.L. Jones for his competent help with electron microscopy, Dr. D.H. Malinowska for her generous supply of rabbit brain, and Mrs. C.A.W. Clarke for typing this thesis.

This work was supported by a Project grant made to Dr. J.R. Lagnado from the Medical Research Council and by a grant-in-aid from Eli Lilly & Co. Ltd. (Basingstoke) who also kindly provided generous supplies of vinblastine and vincristine.

I wish to extend my deep appreciation to my wife for her understanding, encouragement and patience, and I hope that from this work she can, at least, draw some comfort and consolation.

ABBREVIATIONS

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

AMP	Adenosine 5'-monophosphate
ADP	Adenosine 5'-pyrophosphate
ATP	Adenosine 5'-triphosphate
GMP	Guanosine 5'-monophosphate
GDP	Guanosine 5'-pyrophosphate
GTP	Guanosine 5'-triphosphate
ATPase	Adenosine triphosphatase
EGTA	Ethanedioxy-bis (ethylamine)-tetra-acetate
MES	2-(N-morpholino) ethanesulphonic acid
SDS	Sodium dodecyl sulphate
SSA	Sulphosalicylic acid
TCA	Trichloroacetic acid
Tris	2-amino-2-hydroxymethylpropane-1,3-diol

CONTENTS

<u>Chapter</u>	<u>Page</u>
I	INTRODUCTION
1.1.	Historical.....2
1.2.	Characterisation of colchicine-binding (microtubular ) protein.....8
1.3.	<u>In vitro</u> assembly of microtubules.....15
1.4.	Tubulin as a drug receptor.....16
1.5.	Functions of microtubules.....20
1.6.	Clinical investigation.....25
II	GENERAL METHODS
2.1.	Introduction.....29
2.2.	Purification of tubulin.....30
2.3.	Determination of colchicine-binding activity.....41
2.4.	Determination of nucleotide-binding activity.....46
2.5.	Polyacrylamide gel electrophoresis.....47
2.6.	Thin-layer chromatography.....53
2.7.	Protein determination.....55
2.8.	Radioactive counting.....57
2.9.	Expression of data and statistical analysis.....59
III	PURIFICATION AND BINDING PROPERTIES OF FOETAL RABBIT BRAIN MICROTUBULAR PROTEIN
3.1.	Introduction.....62
3.2.	Isolation and characterisation of foetal rabbit brain microtubular protein.....63
3.3.	Comparison of the binding properties of tubulin and of soluble 'transfer factors' isolated from pig brain.....92
3.4.	Phosphorylation of tubulin in vitro and in situ..102
3.5.	Phosphorylation of tubulin-like protein in synaptic membranes.....105
3.6.	Discussion.....109



IV	THE BINDING OF [ <sup>3</sup> H]VINCRIStINE TO TUBULIN	
4.1.	Introduction.....	113
4.2.	Methods.....	113
4.3.	Results.....	132
4.4.	Discussion.....	144
V	THE EFFECTS OF DRUGS ON THE BINDING PROPERTIES OF TUBULIN	
5.1.	Introduction.....	147
5.2.	Effects of drugs on colchicine-binding activity...	147
5.3.	Effects of drugs on GTP-binding activity.....	155
5.4.	Effects of drugs on the aging of colchicine- binding activity.....	157
5.5.	Effects of reserpine on rat brain after <u>in vivo</u> administration.....	159
5.6.	Discussion.....	167
VI	THE EFFECTS OF DRUGS ON THE ASSEMBLY OF MICROTUBULE <u>IN VITRO</u>	
6.1.	Introduction.....	171
6.2.	Methods.....	173
6.3.	Results.....	176
6.4.	Discussion.....	192
VII	DISCUSSION AND CONCLUSION	
(a)	Purification of tubulin.....	199
(b)	Colchicine Binding.....	204
(c)	Nucleotide Binding.....	207
(d)	Vincristine Binding.....	209
(e)	Effects of drugs on binding properties of tubulin.	211
(f)	Conclusion.....	219
	REFERENCES.....	222

Chapter 1      Introduction

1.1      Historical

1.2      Characterisation of colchicine-binding (microtubular)  
protein

1.3      In vitro assembly of microtubules

1.4      Tublin as a drug receptor

1.5      Functions of microtubules

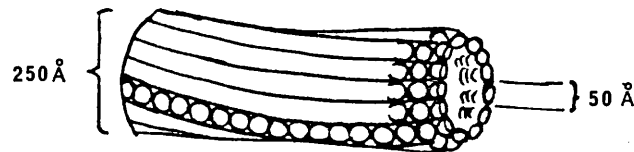
1.6      Clinical investigations

## Introduction

### 1.1 Historical

Microtubules are now recognised as a constant fibrous element present in the cytoplasm of a wide variety of eukaryotic cells. Individual microtubules appear in electron micrographs as straight cylindrical structures, about  $250 \text{ \AA}$  in diameter, consisting of 13 protofilaments, each of which is composed of a linear array of globular subunits about  $50 \text{ \AA}$  in diameter. This is illustrated diagrammatically below:

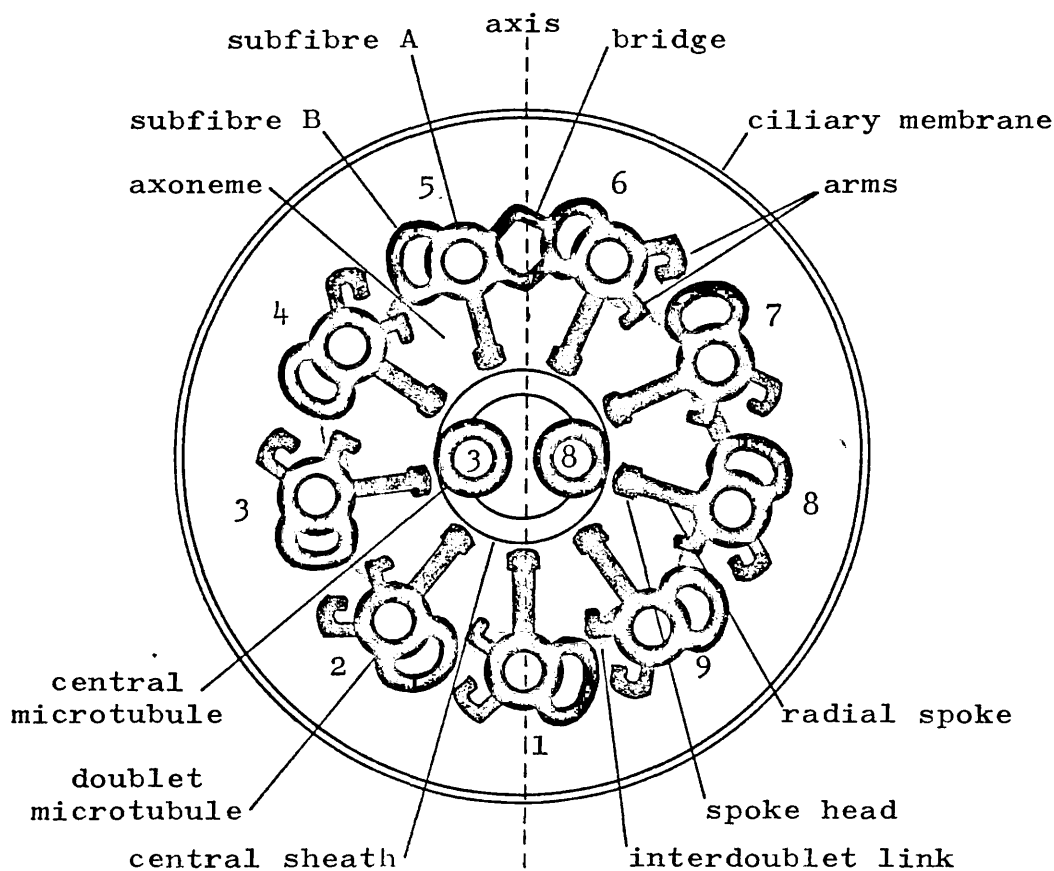
Microtubule



It is worthwhile pointing out, however, that well before the advent of electron microscopy, structures now recognised as consisting of bundles of microtubules or of otherwise interconnected arrays of microtubules, were detected in nerve cells (neurofibrillary apparatus), in dividing cells (mitotic spindle 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).

Stable microtubular structures are the well-known prominent fibrous elements of motile cilia from various organisms, where they are arranged in a characteristic pattern which is referred to as

the "9+2" pattern: each cilium contains 9 pairs of "outer microtubules doublets" near the periphery which are linked to each other through a protein called nexin, and to a spring-like proteinaceous sheath surrounding a "central pair" of microtubules via spoke-like elements. The outer pair of microtubules are referred to as A and B subfibres, and these are connected through the sharing of subunits, common to the wall of each tubule. The complete A subfibre, to which a spoke is attached (one per subfibre), also contains a pair of side-arms which have now been shown to be composed of an ATPase which is referred to as dynein (Satir 1974).



A somewhat simplified version of this pattern is seen in a number of sensory receptor cells, e.g. in the retina rod cells (Sjostrand, 1953), where microtubules are arranged in a "9+0" pattern; this has led to the view that sensory receptors are "modified cilia". In the cutaneous mechanoreceptor cells of the cockroach leg, which are called campaniform sensilia, microtubules have recently been shown to be present in very large numbers, virtually filling the cytoplasm; however, in this case, only occasional inter-tubular links can be detected and in addition, the main functional linkage appears to occur between individual microtubules and the cytoplasmic plasma membrane (Moran & Varella, 1971).

In the case of the mitotic spindle apparatus, again no strict pairing between individual microtubules is apparent, though recent evidence suggests that the numerous microtubules constituting the mitotic spindle are held together, transiently at least, through labile inter-tubular bridges (Hepler et al., 1970). Despite their relative instability, the abundance of microtubules in the mitotic apparatus (e.g. as many as 3,000 per spindle; see Rebhun & Sander, 1967) make it possible to visualise them by electron microscopy even before the use of stabilising agents required to demonstrate the presence of labile cytoplasmic microtubules. However, it was the introduction of glutaraldehyde as a fixing agent in electron microscopy (Sabatini et al., 1963) which finally led to the unequivocal recognition of microtubules as normal cellular "organelles" in a wide variety of eukaryotic cells, at interphase and in particular, in the axonal and dendritic processes of nerve cells, where they are often referred to as neurotubules.

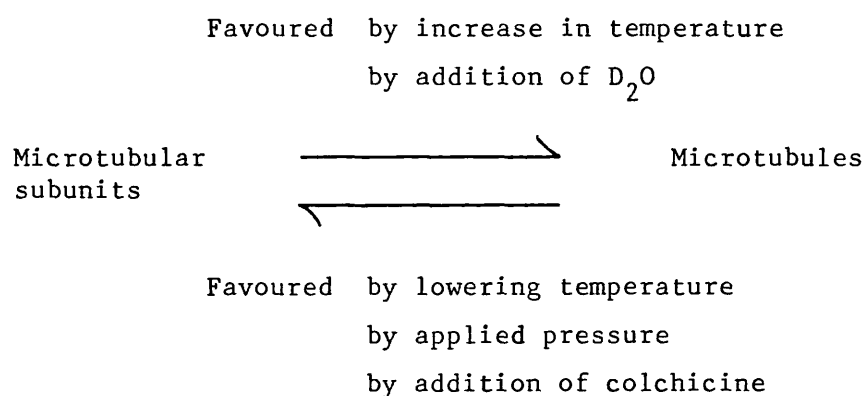
In nervous tissue, microtubules are among the most prominent

linear elements found in axons and dendrites, though they are also found to a lesser extent in the cell body, where indeed most of the protein synthesis in the nerve cell takes place. Interestingly, axonal microtubules appear to begin at the axon hillock<sup>and</sup> to terminate before entering the synaptic terminals (Gray & Guillery, 1966; Wueker & Kirkpatrick, 1972). The density of tubules appears to vary inversely with the cross-sectional diameter of axons (and dendrites) and in very large myelinated axons (e.g. in *Myxicola*, for example, which contains the thickest axon known) microtubules are only rarely detected (Gilbert, 1975). It would appear that the main fibrous elements of large axons are 100 Å diameter argentophilic neurofilaments, which have now been shown to be quite distinct from microtubules in their chemical structure (Huneeus & Davison, 1970). On the other hand, it is apparent that microtubules predominate in thin unmyelinated axons and even more so in the distal thinned portions of the dendritic fibres. There seems, however, to be no obvious geometric organisation of the parallel arrays of microtubules seen even in the thin microtubule-rich regions of dendrites. Nevertheless, it may be of interest that generally speaking, the cytoplasm of the 'receptive' elements of nerve cells (dendrites), which have the highest surface to volume ratio, is often seen to be filled with microtubules (see later).

Although as mentioned above, there is little evidence for structural links between neurotubules with each other or with other cellular elements, it has recently been shown, using lanthanum staining techniques, that the surface of neurotubules in various invertebrate nerve cords seems to be covered with

amorphous "filamentous" extensions which frequently appear to act as inter-tubular links (Burton & Fernandez, 1973). It has also been claimed that axonal neurotubules may form a 3-D network with neurofilaments (Metuzals, 1963).

It is now apparent that the lability of most types of cytoplasmic microtubules may be related to the fact that they exist in a state of dynamic equilibrium with their subunits. This was clearly suggested from the elegant experiments of Inoue (Inoue, 1952; 1964) who studied the effects of various factors on the stability of mitotic spindle microtubular bundles in living cells by birefringence. In particular, he showed that lowering the temperature to below 10°C led to a reversible loss of birefringence and that birefringence increased to a maximum as the temperature was increased to 37°C; furthermore, the antimitotic alkaloid colchicine also acted as a reversible inhibitor of birefringence.



Thermodynamic analysis of these experiments showed that the assembly process was endothermic and proceeded with a large increase in entropy (Inoue & Sato, 1967): these data are similar to those obtained from thermodynamic studies of monomer polymer transformations during the polymerisation of actin (Asakura et al., 1960) and of tobacco mosaic virus coat protein

(Lauffer, 1958).

It was early suggested that the disruption of the mitotic spindle by colchicine and other mitotic inhibitors was due to their interaction with the free microtubular subunits, thereby shifting the equilibrium towards the depolymerised state. Further experiments showed that protein synthesis inhibitors had no effect on the reappearance of birefringence in the mitotic spindle after removal of the mitotic inhibitor, colcemid, by repeated washings (Inoue & Sato, 1967), suggesting that a pool of subunits were readily available for their repolymerisation into mitotic spindle fibres. This led Inoue to propose the theory of dynamic equilibrium, which was also applied to account for the reversible assembly and disassembly of microtubules studied in the axoneme of Actinosphaerium (Tilney & Porter, 1967).

Kinetic studies supporting the theory that colchicine acts through binding (reversibly) to a specific cellular site, thereby initiating the disassembly of the mitotic spindle were later carried out by Taylor, using [ $^3\text{H}$ ]-colchicine (Taylor, 1965). These data led to the view that the rate-limiting step in mitotic arrest was the formation of a colchicine-binding site complex which was tentatively identified as a saturable high-affinity binding site on free microtubular subunits. This stimulated a search for colchicine-binding receptors in various types of cells where microtubules had been observed. It was later found that the kinetics of colchicine binding were the same whether studied in intact cells or in soluble tissue extracts and that the extent of colchicine binding in various tissues correlated well with their mitotic index (Borisy & Taylor, 1967; Wilson & Friedkin, 1967a). However, it was also shown that extracts of adult brain, a typically post-mitotic tissue, were markedly enriched in



colchicine-binding activity as compared to extracts from various other tissues in which interphase cells predominated. This was attributed to the abundance in nerve tissue of cytoplasmic microtubules (Borisov & Taylor, 1967).

The kinetics of colchicine binding in whole cells or in tissue extracts were found to be very similar. In particular it was found that in both types of systems colchicine binding was a slow process which occurred negligibly at 4°C but was maximal at 37°C when tissue was incubated in the presence of micromolar concentrations of labelled colchicine. In each case, saturation of colchicine-binding sites occurred after 1-2 h incubated at 37°C and half-maximal binding occurred at concentrations of colchicine between 1 - 3 μM. This range of concentration corresponds closely to that required for partial or complete mitotic arrest in cultured cells.

## 1.2 Characterisation of colchicine-binding (microtubular) protein

It was early shown that the component responsible for colchicine binding in solubilised central pair microtubules from sea urchin sperm tails or in soluble extracts of brain and cultured Hela cells was a protein which gave a sedimentation coefficient of 6 S during ultracentrifugation and this component was tentatively identified as the globular subunit protein of microtubules (Shelanski & Taylor, 1968; Weisenberg et al., 1968; Wilson & Friedkin, 1967b). Estimates of the molecular weight of the colchicine-binding component from various sources gave value between 110,000 and 120,000. The 6 S component appears to bind between 0.5 and 1 mole of colchicine/

$10^5$  g of protein. This component could be dissociated into monomers of apparent 60,000 molecular weight in the presence of high concentration guanidine hydrochloride or urea. At about the same time, it was shown that the main protein component of microtubules selectively solubilised from *Tetrahymena* cilia contain between 0.5 to 2 mole of guanosine nucleotide/  $10^5$  g of protein (Stephens, 1967), although the form of bound nucleotide was only shown to be GTP or GDP in later work. The amino acid compositions of the microtubular protein isolated from the outer doublets of various species of sea urchin sperm tails or from tetrahymena cilia were shown to be virtually identical (Stephens, 1968); in each case there was a predominant acidic amino acids (glutamic and aspartic). Mohri (1968) gave the generic name tubulin to microtubular subunit protein from various sources after showing that these proteins differ significantly from other fibrous proteins such as actin, myosin and flagellin in their amino acid composition (see also Mohri, 1973).

On the assumption that colchicine-binding is a specific property of microtubular subunit protein, two groups of investigators succeeded in preparing highly purified preparations of tubulin from soluble extracts of brain since this was a particularly rich source of colchicine-binding protein (Adelman et al., 1968; Wilson, 1970). At that time, colchicine binding was the only means available to purify the protein of presumed microtubule origin from tissue such as brain, since the extreme lability of cytoplasmic microtubules did not permit a more direct approach, i.e., the initial isolation of intact microtubules from which subunits could be dissociated. Colchicine-binding protein was purified on the basis of its high affinity for anion exchange

resins (e.g. DEAE Sephadex) from which it could be eluted at relatively high salt concentrations (Weisenberg et al., 1968, Wilson, 1970). The successful purification of the protein was greatly facilitated when it was discovered that the rapid decay of colchicine binding which occurs during purification could be prevented by inclusion of GTP and  $Mg^{2+}$  throughout the isolation procedure. The isolated protein typically contains 1 mole of stably bound guanosine nucleotide (GTP or GDP) per mole of dimer (M.W. 120,000), but can bind up to 1 additional mole of GTP/mole during subsequent incubation with GTP. Using tritiated GTP, it was early shown that this additional guanosine nucleotide-binding site had an extremely short half-life. The two guanosine nucleotide-binding sites are now referred to as the N, or non-exchangeable and the E, or exchangeable sites (Weisenberg et al., 1968). There is now evidence that a transphosphorylation reaction can take place between the terminal phosphate group of GTP loosely bound at the E site and the firmly bound GDP at the N site (Berry & Shelanski, 1972; Jacobs et al., 1974). Little is known as to the functional significance of GTP binding to tubulin (see later, however), although early work had clearly established from competition experiments that guanosine nucleotide and colchicine are bound to different sites of the protein.

Besides the colchicine and GTP-binding sites, brain tubulin as well as other tubulin preparations were found to contain a third and apparently distinct site for the binding of two potent antimitotic alkaloids, vinblastine and vincristine, (Wilson, 1970). These drugs, which are extracted from the leaves of Vinca plants (Beer, 1955; Palmer et al., 1960) are extensively used as anti-tumour agents (Deysson, 1968). Wilson

(1970) showed that low concentrations of vinblastine or vincristine ( $10^{-6}$  -  $10^{-4}$  M) markedly stabilised the colchicine-binding activity of brain tubulin, while other investigations had demonstrated that these alkaloids caused the sequestration and disruption of microtubules in fibroblasts and human leukocytes (Bensch & Malawista, 1969; Bryan, 1972), giving rise to the formation of paracrystalline cellular inclusions later identified as non-tubular aggregates of tubulin (Nagayama & Dales 1970). Recently, Bryan (1974) has demonstrated that crystals induced in the presence of these alkaloids in sea urchin eggs can be isolated from the cells. These isolated crystals, which were shown by electrophoresis to be composed of pure tubulin, were found to contain up to 2 moles of GTP/  $10^5$  g protein and to retain their ability to bind colchicine (Bryan, 1971). Bryan also demonstrated, using labelled vinblastine, that the isolated crystals contained 1 - 2 moles of the alkaloid /  $10^5$  g of protein, i.e. per tubulin dimer.

Other investigators have shown that at relatively high concentrations, vinblastine and vincristine could induce the precipitation of tubulin from soluble extracts of brain (Marantz et al., 1969, Lagnado & Lyons, 1971). Although the vincristine or vinblastine-induced precipitation reaction can be used to obtain partially purified tubulin, other structural proteins such as actin, myosin and membrane proteins can also be precipitated by these drugs under similar conditions (Wilson et al., 1970). Vinca alkaloids appear to act at sites on purified tubulin, or other proteins, which may also selectively bind  $Ca^{2+}$ , and the binding of  $Ca^{2+}$  and vincristine (a base) appeared to be synergistic (Wilson et al., 1970).

More recently, studies have been initiated to investigate the binding properties of tubulin for Vinca alkaloids using tritiated vinblastine and vincristine (Beer et al., 1964; Owellen et al., 1972). Little is known, however, regarding the subcellular distribution of vincristine binding sites in tissue homogenates, but on the basis of the early reports indicating the relative unspecificity of these alkaloids (Wilson, 1970), it seems likely that the distribution of Vinca alkaloid-binding receptors in tissue homogenates may not parallel the distribution of colchicine-binding sites, which are more specifically related to the presence of tubulin. In this connection, however, it is worth noting that a considerable portion of the colchicine-binding activity present in brain homogenates is tightly associated with particulate fractions, especially with components of isolated synaptosomal structures, as was first shown by Feit & Barondes (1970) and Lagnado et al., (1971), and that recent work suggests that tubulin may be a major structural component of synaptic junctional complexes (Walters & Matus, 1975; Lagnado et al., 1975).

Recently, more detailed investigations on the properties of chromatographically purified brain tubulin showed that in the presence of urea, the protein can be dissociated into two closely related components during electrophoresis on SDS-polyacrylamide gels. The two forms of tubulin monomer, which are now referred to as the  $\alpha$  and  $\beta$ -subunits, gave apparent molecular weights of 56,000 and 53,000, respectively, on the basis of their mobility in SDS-polyacrylamide gels (Feit et al., 1971; Olmsted et al., 1971).

However, there is now evidence that the separation of tubulin into two distinct bands may not in fact represent a difference in

molecular weight between the  $\alpha$  and  $\beta$  forms, but rather that this anomalous behaviour (seen under restricted conditions of electrophoresis) may reflect a difference in charge between two polypeptides of the same molecular weight (Bryan, 1974; see also Discussion, Chapter 7).

The above view would be in keeping with the small differences found in the amino acid composition of the  $\alpha$  and  $\beta$  forms of tubulin (Bryan & Wilson, 1971). There is now substantial evidence that the colchicine-binding dimers of tubulin are  $\{\alpha\beta\}$  heterodimers (Bryan & Wilson, 1971; Wilson et al., 1974).

The small differences in amino acid composition between the  $\alpha$  and  $\beta$  forms of tubulin are also reflected in the peptide maps obtained after trypsin digestion (Feit et al., 1971). Very recently, it has been demonstrated that the  $\alpha$  and  $\beta$  forms of tubulin purified from sea urchin sperm tails or from brain have methionine as the N-terminal amino acid; furthermore, on the basis of partial sequence data, small differences in the amino acid sequence within the first 25 amino acids (starting at the N-terminal Met) are apparent (Luduena & Woodward, 1973). The additional observations based on limited sequence data further indicate that the homology between  $\alpha$ -tubulins or between  $\beta$ -tubulins derived from brain and sea urchin egg is greater than that between  $\alpha$  and  $\beta$  tubulin from any given species. From this it was concluded that the  $\alpha$  and  $\beta$  forms may originate from a common ancestral gene and that both forms of tubulin are among the most conservative proteins known. This extreme conservatism in structure is not so surprising if one considers the many specific binding requirements involved in ligand recognition or in protein-protein interactions necessary for microtubule formation which are common to microtubular proteins from widely diverse sources.

In addition to the various binding properties of tubulin which have already been described, it was recently found that tubulin purified from brain contains between 0.5 - 1.0 mole of protein-bound phosphoserine phosphate per mole of dimer (Reddington & Lagnado, 1973, Eipper, 1974; Lagnado et al., 1975). This is in keeping with earlier evidence that brain tubulin possesses a cyclic AMP-dependent protein kinase: incubation of purified tubulin in the presence of  $\{\gamma^{32}\text{-P}\}$  ATP and  $\text{Mg}^{2+}$  results in the incorporation of  $\{^{32}\text{P}\}$  into protein-bound serine residue of tubulin (Goodman et al., 1970; Lagnado et al., 1972; Soifer et al., 1972). Interestingly, in vitro phosphorylated protein, unlike the relatively unphosphorylated protein, appears to readily interact with a heat-labile factor present in brain supernatant, as a result of which, the phosphorylated protein can be precipitated at a relatively low concentration of ammonium sulphate and it is not absorbed onto DEAE Sephadex (Murray & Froscio, 1971). It was recently shown that tubulin can also be phosphorylated in situ after incubation of diced rat brain (Eipper, 1972) or guinea pig brain slices (Reddington & Lagnado, 1973) with  $\{^{32}\text{P}\}$  orthophosphate. Furthermore, Eipper (1974) has reported that the phosphorylated peptides obtained after tryptic digestion of in vitro phosphorylated tubulin were different from those obtained with tubulin phosphorylated in vivo

It is not clear, however, whether the "intrinsic" protein kinase activity in isolated tubulin preparations is a property of tubulin itself or of a closely associated minor protein copurified with tubulin. The kinase(s) responsible for the phosphorylation of tubulin in situ has not been identified. It is of interest, however,

that a tubulin-like protein recently identified in isolated synaptic membrane preparations is also a substrate for a membrane-associated protein kinase (Lagnado et al., 1975).

Most of the biochemical properties of tubulin described above have been derived from chromatographically purified tubulin, i.e., a purification procedure which selectively purified colchicine-binding protein. Although early comparisons of the electrophoretic behaviour and binding properties of colchicine-binding protein from brain appear on the whole to differ little from those described for microtubular subunit protein derived more directly from isolated "stable" microtubules (e.g. from cilia). Attempt to polymerise colchicine-binding protein into microtubules in vitro has only recently been achieved. Recent work concerning the reassembly of microtubules in vitro has now unequivocally shown that the colchicine-binding protein dimer is, indeed, a subunit of microtubules.

### 1.3 In vitro assembly of microtubules

The key discovery that led to the successful reassembly of microtubules in vitro was made by Weisenberg, (1972) who showed that under conditions which maintained a low concentration of free  $\text{Ca}^{2+}$  (i.e. in the presence of EGTA), incubation of crude soluble extracts of brain at  $37^{\circ}\text{C}$  in the presence of GTP or ATP resulted in the rapid formation of readily recognisable microtubules. Microtubules formed in this way were stabilised by the inclusion of high concentrations of glycerol or sucrose in the assembly buffer and under these conditions, polymerisation could take place even in the absence of added ATP or GTP (Shelanski et al., 1973). The fact that the polymerisation process was inhibited by colchicine



and was highly temperature-dependent fits in very well with what was known concerning the polymerisation of microtubules in living cells (Inoue & Sato, 1967). Using viscometry, coupled with electron microscopy to follow the assembly of microtubules in vitro, Borisy et al., (1972) have shown that the assembly process is markedly inhibited when GTP is replaced by GDP or by the non-hydrolysable analogue of GTP,  $\beta$ , $\gamma$  methylene guanosine triphosphate (GMPPCP), suggesting that the hydrolysis of terminal phosphate group is some way involved in the assembly of microtubules.

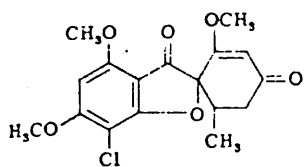
In all these studies, it was clearly shown that tubulin ( $\alpha$ ,  $\beta$  forms) accounted for 85 - 90 % of the protein derived by disassembly in the cold of pelleted microtubules which had been reassembled in vitro. The additional observation was made that the assembly process appeared to depend initially on the presence of 36 S polymers of tubulin which apparently act as nucleating sites. These small polymers can be sedimented after high speed (230,000 g x 90 min) centrifugation and electron microscopic examination of the resulting pellet shows that they appear as disc-like structures 200 - 400 Å in diameter (Borisy & Olmsted, 1972, Kirschner et al., 1974).

#### 1.4 Tubulin as a drug receptor

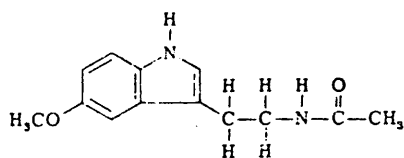
There are a great number of spindle poisons of which colchicine is probably the most well known. It has been known for a long time that colchicine inhibits spindle formation, thereby preventing the separation of daughter chromosomes at anaphase, and this has now been related to the binding of this drug to spindle microtubular subunits (see above).

Fig.1.1.

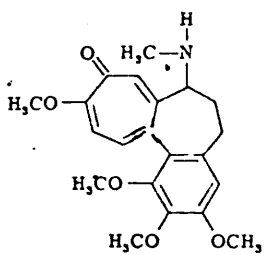
## Chemical Formulae of Antimitotic Drugs



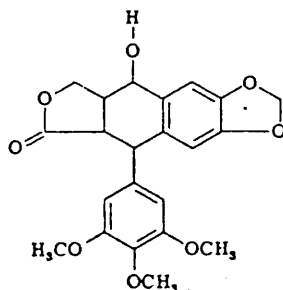
Griseofulvin



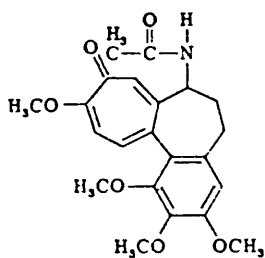
Melatonin



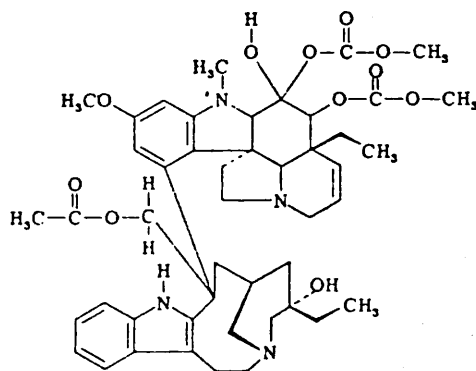
Colcemid



Podophyllotoxin



Colchicine



Vinblastine

The specificity of colchicine as an antimitotic agent has been extensively investigated by Deysson (1968). It was shown that ultraviolet irradiation of colchicine, which results in the splitting of the 7-membered ring, abolishes its antimitotic effect; the irradiation-induced  $\beta$  and  $\delta$  isomers of luminocolchicine were found to be inactive as antimitotic agents (Wilson et al., 1974). Other antimitotic drugs such as podophylotoxin have now been found to compete with colchicine for the same binding site of brain tubulin (Wilson, 1970). Griseofulvin, another spindle poison has been found to exert antimitotic effects similar to colchicine though it does not inhibit the binding of colchicine or Vinca alkaloids to tubulin, suggesting that its action may not directly involve interaction with microtubular protein (Wilson, 1970). In studying the effects of various antimitotic alkaloids on regeneration of the Stentor membranelle band, which consists mainly of microtubules, Margulis<sup>1972</sup> has shown that a large number of heterocyclic compounds which contain a methoxy group with a nearby negative atom such as -OH or -NH group in the molecule, seem to inhibit the formation of microtubules. It is worth noting that many of the 'anti-tubulin' drugs such as colchicine and the Vinca alkaloids fulfil this structural requirement (see Fig. 1.1 for chemical structures and Discussion in Chapter VII). It has recently been reported that tropolone, which consists of only a 7-membered ring with a hydroxyl group and a carbonyl group, exerts an antagonising effect on colchicine in vivo (i.e. tropolone reverse metaphase arrest produced by colchicine in fibroblast cultures (Benitez et al., 1954)), whereas kinetic studies on the effects of tropolone on the binding of colchicine to calf brain tubulin showed a reverse effect i.e., tropolone stabilised colchicine binding to tubulin (Kalra & Fragata, 1974). This dual effect of

tropolone is of great interest but remains to be explained.

The specific binding of colchicine, and to a less extent of Vinca alkaloids, to microtubular protein is being used as an investigative tool for studying the various cellular functions which may be directly or indirectly related to microtubules. Recent evidence shows that colchicine does not seem to affect intact microtubules and this suggests that the colchicine-binding sites must be blocked or the affinity substantially decreased in the assembled microtubules (Wilson et al., 1974). In addition to their effects as inhibitors of chromosome translocation at anaphase, both colchicine and Vinca alkaloids have been reported to inhibit a great variety of physiological processes such as axoplasmic transport (Dahlstrom, 1968; Banks et al., 1971), the secretion of hormones from a number of endocrine glands (Williams & Wolff, 1970; Temple et al., 1972), and the outgrowth of neurites of neuroblastoma cells (Seeds et al., 1970). It is generally assumed that colchicine and the Vinca alkaloids inhibit these cellular processes by acting directly on microtubule assembly systems. However, it is possible that these drugs could also act indirectly via binding to other cellular sites, affecting membrane transport (Wilson et al., 1974). Furthermore, recent evidence that tubulin is a significant component of the synaptic membrane suggests that in this tissue at least, the disruption of neural function by these drugs could be related to their interaction with non-tubular forms of microtubular protein (Lagnado et al., 1971, 1975; Chapter III).

### 1.5 Functions of microtubules

The widespread occurrence of microtubules in cells and organisms indicates that they are involved in various functions. The presence of long microtubules throughout elongated cells such as nerve cells suggests that not only do they provide cytoskeletal support to the cellular processes, they may also act as conveying belts for transporting structural, functional and metabolic substances synthesised in the cell body to the distal ends of the cell for growth, maintenance and function of the cell. The unique paired microtubules with their characteristic arrangement along the whole length of cilia, flagella, axopods and sperms tails signify their involvement in the motility of these active structures. The movement of chromosomes during cell division is also dependent on the integrity of the spindle microtubules. There is much more limited evidence that microtubules may also partake in processes associating with sensory transduction and with the release of hormones and neurotransmitters.

#### Cytoskeletal

The destruction of microtubules in the axopods of Actinosphaerium seen after lowering of temperature (Tilney & Porter, 1967), applying high pressures (Tilney, 1968), or after the addition of colchicine (Tilney et al., 1968) or urea (Shigenaka et al., 1971) resulted in retraction of the axopods.  $D_2O$  which has been found to stabilise spindle microtubules, prevented the pressure and cold-induced retraction of axopods. It has also been reported that the addition of colchicine to growing nerve cells in tissue cultures stopped the elongation of axons without affecting the growth cone (Seeds et al., 1970; Yamada et al., 1970). Colchicine and vinblastine also prevented the elongation of developing lens tissue (Arnold, 1966).

These observations support the role of microtubules in formation and maintenance of assymmetric cell structures.

#### Axoplasmic transport

The possible involvement of neurotubules in axoplasmic transport stimulated a vast amount of research. The only ultrastructural evidence for a role of neurotubules in the axoplasmic transport has been obtained from studies on the central nerve cord of the sea lamprey, where a close association between microtubules and synaptic vesicles has been observed, suggesting that neurotransmitter-containing vesicles assembled in the cell body may be transported to nerve endings through a mechanism involving association with microtubules. (Smith et al., 1970; Smith, 1971) It has been shown that axoplasmic transport occurs at two distinct rates in nerve fibres. Although the rate of transport varies according to the biological system under investigation, the slow rate is usually taken to be below 20 mm/day while the fast rate may vary from 20 to 500 mm/day (Jeffrey & Austin, 1974).

Colchicine has been shown to induce a blockage of axoplasmic transport in a wide variety of nerve preparations in both the fast and slow transport systems. The rapid transport of protein in the rabbit optic system (Karlson & Sjostrand, 1969), in the hypoglossal and vagus nerves of rabbit (Sjostrand et al., 1970) and the slow transport of material in chicken sciatic nerve (James et al., 1970) were all found to be sensitive to colchicine. Vinblastine also blocks the axoplasmic transport of various neural preparations. When high concentrations of vinblastine and colchicine were added to

sympathetic ganglia, as accumulation of noradrenaline as detected by fluorescence was observed (Dahlstrom, 1968). When these drugs were injected under the perineurium of sciatic nerve an accumulation of catecholamine was detected above and at the site of injection and these effects were suggested to be due to the inhibition of the rapid transport of catecholamine storage granules through disruption of the neurotubules.

Several theories involving neurotubules have been put forward to explain the mechanism of axoplasmic transport. Schmitt (1968) has speculated that neurotubules act as stationary structures in which bound GTP or ATP may be hydrolysed during interaction with vesicle-bound nucleoside triphosphatases, and that the energy released during the reaction would result in conformational changes in the protein structure of the organelle membrane, thereby providing a motive force to move the particle along the microtubule. However, there is no direct experimental evidence for this view.

From observations on crayfish ventral nerve cord, in which microtubules are found to be coated with a layer of mucopolysaccharide, Samson (1971) has suggested that the anionic residues due to the presence of branching polysaccharide units could be involved in specifically binding macromolecules or organelles and that by a sequence of contraction and expansion of the polysaccharide polyelectrolyte, particles could be moved along the surface of microtubules. Ochs (1971) has presented a "sliding filament" theory in which particles which have been synthesised in the cell body can bind to the neurotubules and neurofilaments by means of cross-bridges: an exchange of cross-bridges would then translocate particles along the fibres. In common with the

polyelectrolyte model of Samson, the energy required for the exchange between the cross-bridges would be derived from the hydrolysis of ATP, as judged from the inhibitory effects of respiration and oxidative phosphorylation inhibitors on axoplasmic transport seen in mammalian nerves (Ochs, 1971)

At present, none of these theories adequately <sup>explain</sup> various aspects of axonal transport. Furthermore, it is not clear whether the retrograde transport of viruses (Kristensson, 1970), horseradish peroxidases (Kristensson, 1971) or other exogenous macromolecules in axons also involves microtubules.

#### Motility

The role of microtubules in the movement of cilia and sperm tail flagella is more apparent. It has been shown that microtubules in cilia slide along one another by means of their dynein side-arms which contain ATPase activity and that the hydrolysis of ATP by this enzyme provides the required energy for the movement (Satir, 1974). It has also been shown that the disruption of cytoplasmic microtubules by colchicine led to a loss of oriented movement and extension of pseudopodia in cultured cells (e.g. fibroblasts) further supporting the view that microtubules are involved in motility (Goldman, 1971).

#### Sensory transduction

The association of cilia with sensory receptors such as olfactory cells (Reese, 1965), vertebrates rods and cones (Sjostrand, 1953) and insects receptors (Gray & Pumphrey, 1958)



gave rise to the suggestion that microtubules are involved in sensory transduction. Friedman (1971) recently observed a close 'functional' association of microtubules with the plasmalemma in a sensory organ of the cricket. More direct evidence comes from the work of Moran & Varela (1971) who showed that the application of colchicine or vinblastine to the mechanoreceptor of cockroach leg (campaniform sensillum) resulted in the disruption of microtubules and a failure to evoke action potentials in these receptors.

#### Hormone and transmitter release

The release of insulin from  $\beta$  cells (Lacey *et al.*, 1968), of thyroxin and iodine secretion from thyroid gland (Williams & Wolff, 1970) and of catecholamines from adrenal medulla (Poisner & Bernstein, 1971) has been found to be inhibited by colchicine and this has been taken as evidence for the involvement of microtubules in the secretion of hormones and neurotransmitters.

Various lines of evidence suggest that the blockage of synaptic transmission by colchicine is due to its disruptive effect on axoplasmic transport (Perisic & Cuenod, 1972; Robert & Cuenod, 1969). However, various investigators have also shown that colchicine and other antimitotic drugs may also be acting on the nerve terminal membrane. By analysing the effects of these drugs on the end-plate potential (epp) of frog sartorius muscles, Katz (1972) has shown that colchicine and podophyllotoxin decreased the mean quantal content of end-plate potentials, suggesting that they are acting on the synaptic membrane. Similar work by Turkanis has shown that colchicine and vinblastine produced a significant (approximately

50 %) decrease in the amplitude of miniature end-plate potentials (mepp) in the same type of neuromuscular junction preparation. Recent evidence for the presence of tubulin as a significant component in the synaptic plasma membrane further supports a functional role of microtubular protein in the release of transmitter substances (Lagnado et al., 1975 ).

#### 1.4 Clinical investigations

A disease which<sup>is</sup> characterised histologically by the degeneration of neurites had been observed as early as 1907 by Alzheimer (see Kidd, 1963) and is now known as Alzheimer's disease. Since then this disease has been investigated in greater detail and studies of the pathological neurons by electron microscopy revealed the presence of bundles of paired twisted microtubules corresponding to the thickened neurofibrils originally observed by light microscopy (Kidd, 1963; 1964). In common with Alzheimer's disease, the accumulation of bundles of abnormal microtubules has also been observed in the brain of patients suffering from senile dementia (Terry & Wisniewski, 1970), from postencephalitic Parkinsonism (Wisniewski et al., 1970), from Guam-Parkinson dementia (Hirano et al., 1968) and from Pick's disease (Schochet et al., 1968). Since these twisted microtubules have never been isolated and studied, it is not known whether these abnormal microtubules are derived from normal microtubules or whether they are formed from aberrant forms of protein subunits only present in the nerve cells of pathological tissues.

Colchicine, vinblastine and podophyllotoxin have been reported to cause the disappearance of microtubules in vivo and the

proliferation of 'neurofilaments' (100 Å thick); this evidence led to the suggestion that both 'neurofilaments' and neurotubules shared the same protein subunit and that interconversion between the two structures could take place (Wisniewski et al., 1968). Recently, however, neurofilaments (90 Å in diameter) have been isolated from bovine brain and their subunit was found to have physico-chemical properties different from those of tubulin; it has molecular weight of about 60,000 and it neither co-migrates with tubulin nor binds colchicine or nucleotides. (Shelanski et al., 1971.) It is therefore unlikely that drug-induced 100 Å filaments are homologous to normally occurring neurofilaments of the same diameter.

The accumulation of neurofilaments ('neurofibrillary degeneration') has been detected in neural tissue under a wide range of conditions, for example, in Pick's disease (Terry & Pena, 1965), colchicine and vincristine encephalopathy (Wisniewski & Terry, 1968), Vitamin E deficiency (Lampert et al., 1964), copper deficiency (Cancilla & Barlow, 1966), acrylamide neuropathy (Prineas, 1969) and Wallerian degeneration (Gray & Guillery, 1966). The proliferation of 'neurofilaments' is probably due to a non-specific change associated with neuronal injury. A recent study on human brain tubulin shows that its biochemical and pharmacological properties are similar to those of other preparations obtained from non-human sources (Eng et al., 1974). Further investigation on the properties of tubulin isolated from abnormal microtubular structures of pathological tissue might provide a clue as to the pathogenesis of human brain diseases associated with microtubular malformation.

Chapter II      General Methods2.1      Introduction

## 2.1.1      Chemicals

## 2.1.2      Preparation of crude tissue extracts

2.2      Purification of tubulin

## 2.2.1      Batchwise method

## 2.2.2      Gradient elution method

## 2.2.3      Polymerisation method

2.2.4      Precipitation with Vinca alkaloids2.3      Determination of colchicine-binding activity

## 2.3.1      Incubation conditions

## 2.3.2      Column chromatographic assay using Sephadex G100

## 2.3.3      Batchwise DEAE-A50 Sephadex chromatographic assay

## 2.3.4      DE 81 filter disc assay

## 2.3.5      Comparison of methods

2.4      Determination of nucleotide-binding activity

## 2.4.1      Incubation mixture

## 2.4.2      Millipore filtration assay

2.5      Polyacrylamide gel electrophoresis

## 2.5.1      General procedure for preparation of gels

## 2.5.2      Buffer systems for electrophoresis

## 2.5.3      Preparation of samples and electrophoresis

## 2.5.4      Fixing, staining and destaining

## 2.5.5      Molecular weight determination

## 2.5.6      Slicing and drying of gels for radioautography

2.6      Thin-layer chromatography

2.6.1      Colchicine

2.6.2      Nucleotides

2.7      Protein determination

2.8      Radioactive counting

2.8.1      Preparation of radioactive chemical solutions.

2.9      Expression of data and statistical analysis

## Chapter II      General Methods

### 2.1      Introduction

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

#### 2.1.1      Chemicals

All 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. Unlabelled nucleotides were purchased from Boehringer Corporation Ltd. Vincristine and Vinblastine were a gift from Eli Lilly Co. Ltd., Basingstoke, U.K. Other drugs used will be specified in the text.

The purity of labelled drugs and nucleotides was periodically checked by thin-layer chromatography as described in section 2.6, and appropriate corrections were made in the determination of specific radioactivities, in the case of labelled 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 tissue extracts

Brains excised from etherised animals (rabbit, rat, guinea pig) were kept on ice and freed of blood vessels and meninges. The weighed tissue was homogenised in 9 vol of an ice-cold solution of

10 mM - Na phosphate buffer (pH 6.8), containing 10 mM -  $MgCl_2$  and 0.24 M - sucrose, by 7 up-and-down strokes, using a motor-driven glass homogeniser with Teflon pestle (clearance 0.003 - 0.005 in). For preparation of tubulin, the homogenate was centrifuged for 1 h at 100,000 g to yield a soluble fraction from which tubulin was isolated, and a particulate fraction which was resuspended in the homogenising medium.

## 2.2 Purification of tubulin

Tubulin was prepared from the brain of various species such as rabbit, guinea-pig and rat. However, for most of the experiments to be described, tubulin was purified from foetal rabbit brain, since this material, which was readily available, proved to be a rich source of the protein. The whole purification procedure was carried out between 2 - 4° C unless otherwise specified. Early methods employed were based on the original procedure of Weisenberg et al. (1968) (see 2.2.1. and 2.2.2), but these were later superseded by a more direct method of purification in which tubulin was derived from microtubules reassembled in vitro (see 2.2.3). In addition, tubulin was occasionally partly purified by precipitation with Vinca alkaloids (see 2.2.4).

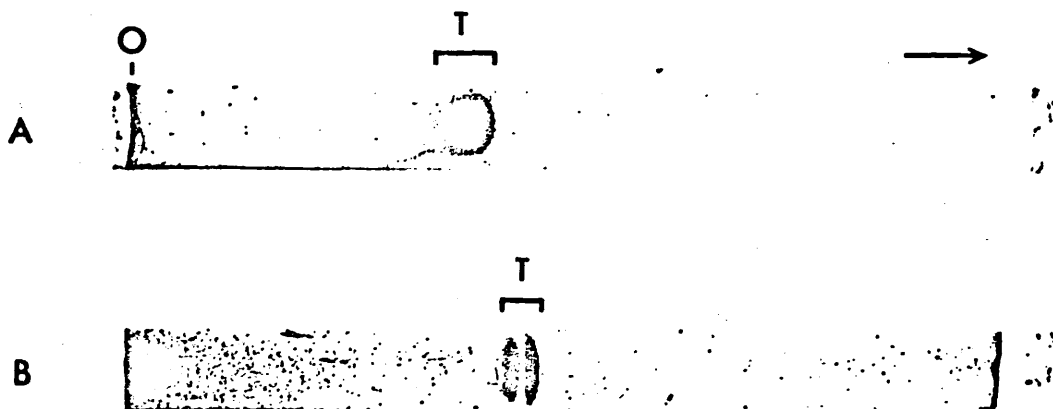
### 2.2.1 Batchwise method

In this method, tubulin was purified from foetal rabbit brain by the procedure of Karlsson & Sjostrand (1971) which is an adaptation of the original method of Weisenberg et al., (1968) for use with small amounts of starting material.

Brains (ca. 5g fresh weight, from 6 animals) were quickly excised

**Fig.2.1.** SDS-Polyacrylamide gel electrophoresis patterns of chromatographically purified foetal rabbit brain tubulin.

---



A: phosphate buffer system

B: tris-glycine buffer system + urea

For details, see section 2.5. and Chapter III, 3.2.2.

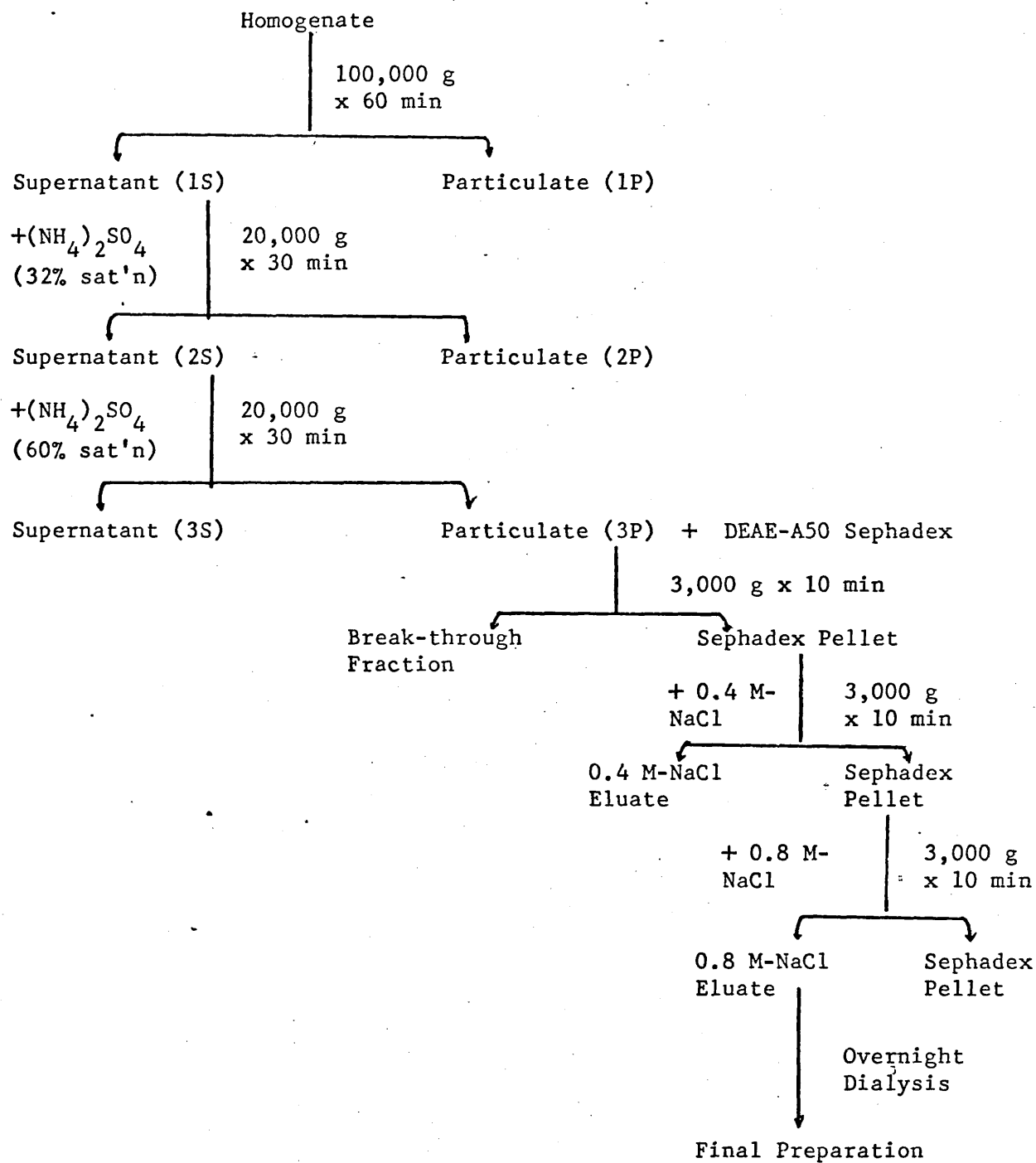
T = tubulin



Figure 2.2.

Scheme for purification of tubulin by batchwise procedure

(see text for details)



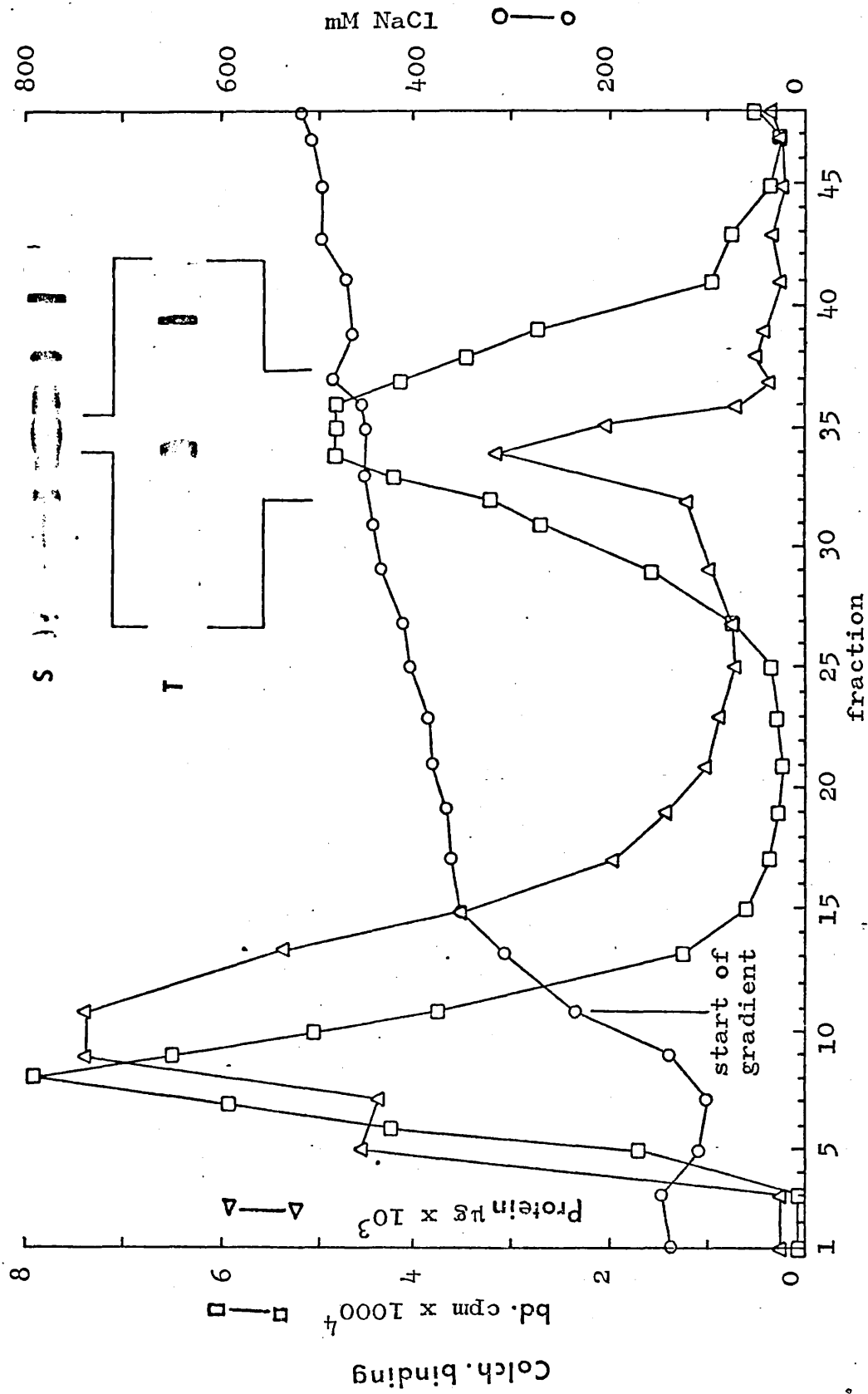
from rabbit foetuses taken from a 28-day pregnant female rabbit (New Zealand White), which had been anaesthetised by injection of nembutal into the ear vein and lumbar region. The brains were kept on ice and adhering blood vessels and meninges were teased away. After weighing, the tissue was rinsed with ice-cold 10 mM - Na - phosphate buffer, pH 6.8, containing 10 mM-MgCl<sub>2</sub> and 10 mM-GTP (P-Mg-GTP buffer) and then homogenised with one part of ice-cold P-Mg-GTP buffer containing 0.24 M sucrose (P-Mg-GTP-sucrose buffer). The tissue was homogenised in a motor-driven Teflon-glass homogeniser, by applying eight up-and-down strokes. The homogenate was centrifuged at 100,000 g for 1 h in an MSE SS40 Superspeed centrifuge. The resulting supernatant (designated 1S) was brought up to 32 % saturation with solid ammonium sulphate (177g/l) (BDH, enzymic grade) added over a period of 30 min during which the solution was gently stirred by means of a magnetic stirrer. The first ammonium sulphate precipitate (designated 2P) was removed by centrifugation at 20,000 g for 30 min and the supernatant (2S) brought up to 60 % saturation (390 g/l) with solid ammonium sulphate over 30 min. The precipitated protein (3P) was collected by centrifugation at 20,000 g for 30 min and redissolved in P-Mg-GTP buffer (about 1 ml for every 4 ml of the initial high-speed supernatant). This was mixed with an equal vol of packed DEAE A50 Sephadex (Pharmacia Co.) which had been previously treated as recommended by the manufacturers and pre-equilibrated with P-Mg-GTP buffer. After standing for 30 min, the suspension was centrifuged at 3,000 g for 10 min and the Sephadex pellet was eluted with an equal vol of P-Mg-GTP buffer containing 0.4M-NaCl. Elution was carried out over a period of 30 min with intermittent stirring. The Sephadex was again pelleted by low speed centrifugation and eluted twice with P-Mg-GTP buffer containing 0.8 M-NaCl, each time

with the same vol as was used during the first elution step. The tubulin-rich 0.8M-NaCl eluate was then rapidly dialysed in a rotating dialyser against 500 vol of P-~~M~~<sup>a</sup>-GTP buffer for at least three hours in order to lower the concentration of NaCl which would otherwise inactivate colchicine-binding activity (see 2.2.2). Samples of each fraction were assayed for colchicine-binding activity by DE 81 filter disc method (2.3.4) and analysed by polyacrylamide gel electrophoresis (see Fig.2.1. For method see 2.5 and for data see Chapter III.3.2.2.). The whole procedure is summarised diagrammatically in Fig.2.2)

#### 2.2.2 Gradient elution method

This is a modified version of the batch-wise method and preliminary studies were carried out in order to establish its usefulness as a quick method for isolating tubulin. Samples of the initial high-speed supernatant fraction (IS) or of the redissolved second ammonium sulphate pellet (3P), containing 5-10 mg of protein/ml, were first incubated with ( $^3\text{H}$ ) colchicine for 90 min at 37°C (see later section). After cooling, the mixtures were loaded directly onto a column (2.5 x 6 cm) of DEAE A50 Sephadex which had been pre-equilibrated with 20mM-sodium phosphate buffer, pH 6.8, containing 100 mM-NaCl (see Bryan & Wilson (1971)). The column was initially eluted with 20 ml of this buffer, and another 50 ml of the same buffer containing 400 mM-NaCl. This was followed by a linear NaCl gradient (400 mM-800 mM NaCl) in 20 mM-sodium phosphate buffer, pH 6.8. Two ml fractions were collected by means of a fraction collector and the linearity of the NaCl gradient was monitored by estimating the concentration of chloride ions in each fraction using an EEL chloride

Fig.2.3. Chromatographic purification of tubulin from foetal rabbit brain on DEAE-A50 Sephadex column.



Details of experiment are described in the text, section 2.2.2.

Inset photographs show protein electrophoretic patterns for the pooled fractions 33-36 eluted during chromatography.

T = tubulin. S = initial supernatant.

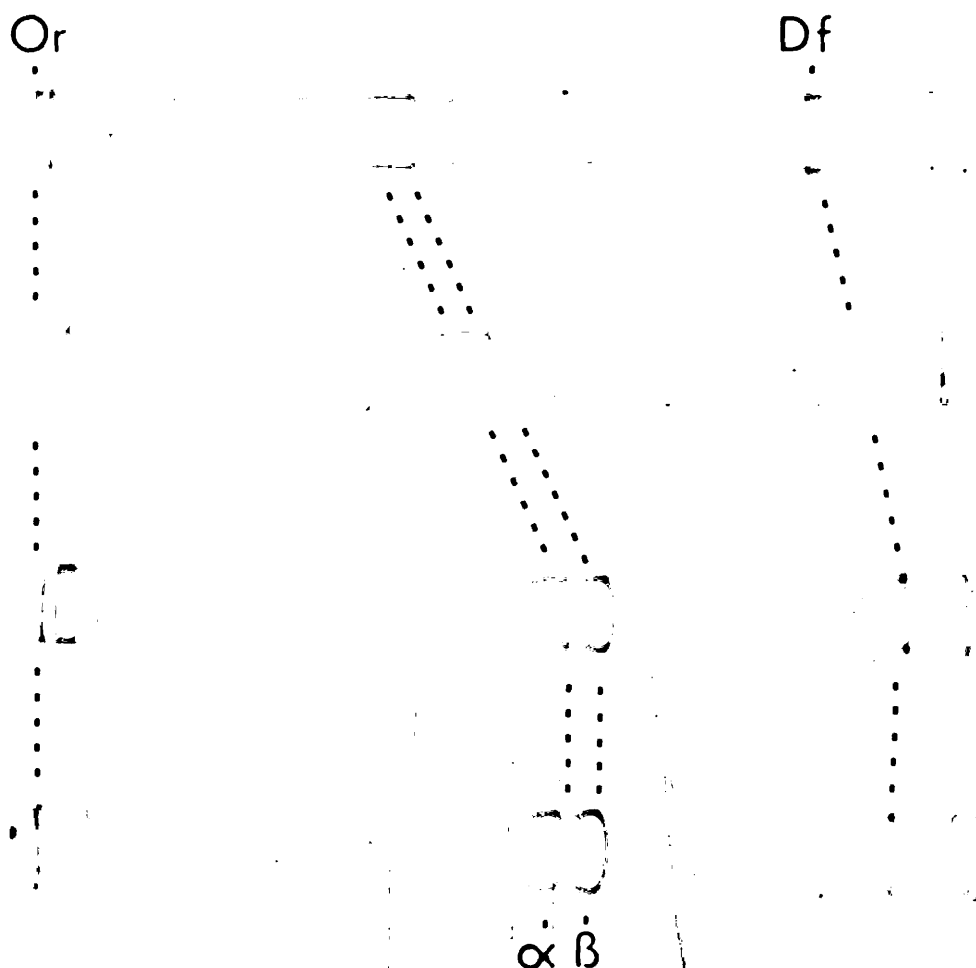
meter. A sample from each fraction was counted for radioactivity, while the protein content was estimated by measuring the absorbance at 280 nm in a Beckman spectrophotometer. A typical elution profile of the purification is shown in Fig.23. Fractions which constituted the protein peaks were pooled and assayed for colchicine-binding activity and concentrated samples obtained by precipitation with 60 % ammonium sulphate saturation, were analysed by polyacrylamide gel electrophoresis (see Fig.23).

### 2.2.3 Polymerisation method

In this method, tubulin was isolated from microtubules assembled in vitro by the method as described by Shelanski et al. (1973). Foetal rabbit brains, freed from adhering meninges and blood vessels, were homogenised at 4°C with 1.5 vol of 0.1 M-2-(N-morpholino) ethanesulphonic acid (MES) buffer, pH 6.5, containing 1 mM-ethyle neglycolbis-(aminoethylether) tetra-acetic acid (EGTA); 1 mM-GTP and 0.5 mM-MgCl<sub>2</sub>. The homogenate was centrifuged at 100,000 g for 1 h at 4°C and the resulting supernatant (SI) was mixed with an equal vol of 'assembly' buffer, consisting of MES buffer containing 8 M-glycerol. The mixture was incubated for 30 min at 20°C yielding a gelatinous pellet (P2) which was found to be enriched in microtubules, but which also contained non-tubular aggregates as seen by electron microscopy (see Chapter VI, 6.3.1. ). The pellet (P2) was then resuspended in a vol of cold MES buffer equivalent to about one third of the vol of the first supernatant (SI) fraction by gentle homogenisation (3 strokes) in a Teflon-glass homogeniser. After standing on ice for 30 min in order to depolymerise microtubules, the suspension was centrifuged at 100,000 g for 1 h at 4°C to sediment any remaining particulate material (P3). The clear

Fig 2.4.  
 Polyacrylamide gel electrophoresis patterns of  
 samples obtained during purification of  
 microtubular protein from foetal rabbit brain  
 by the in vitro assembly method

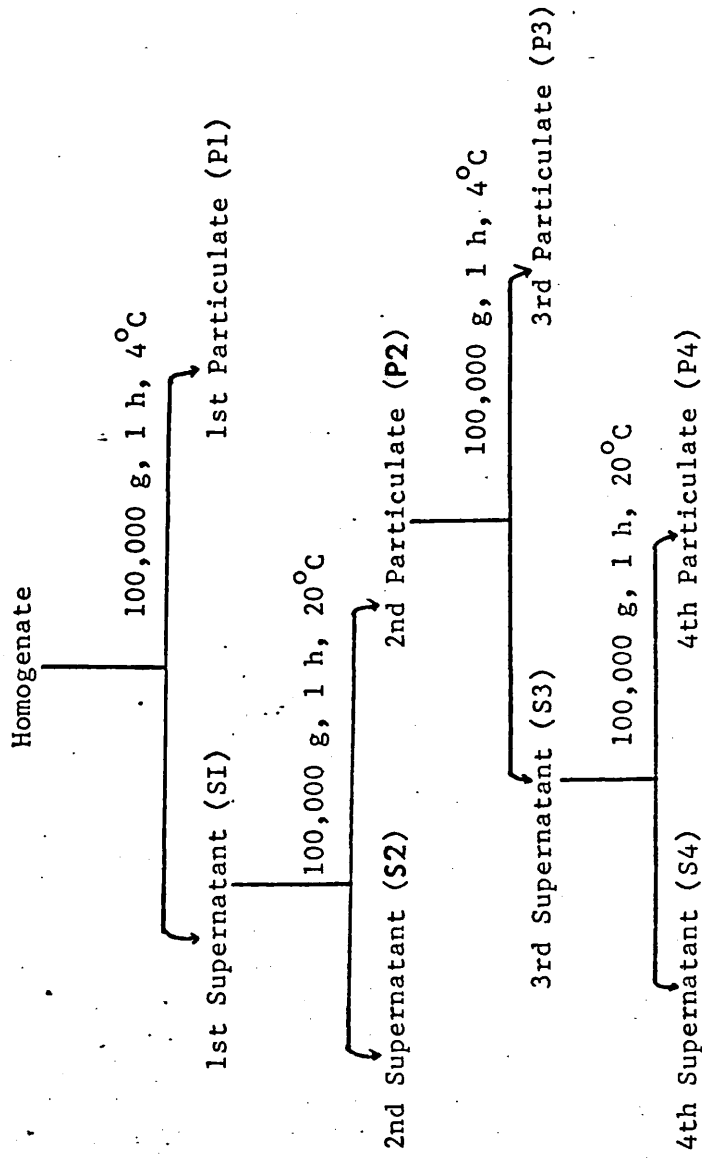
---



Electrophoresis was carried out in tris-glycine buffer system in the presence of urea (see 2.5.).  
 S1 = initial high speed supernatant  
 Fraction P 2, P 3 & P 4 refer to pellets obtained subsequently as indicated in Fig. 2.5. (see also text, 2.2.3).  
 P 4 = final microtubular protein pellet. Note minor high molecular weight components near origin (Or) as well as main tubulin bands ( $\alpha\beta$ ). Df = dye front.

Fig. 2.5.

Procedure of tubulin purification by polymerisation method.



supernatant (S3) was then mixed with an equal vol of MES buffer containing 8M-glycerol and incubated for 30 min at 37°C to yield a pellet (P4) consisting mainly of polymerised microtubules as judged by electron microscopy (see Chapter VI, Fig.6.2). The purity of the preparation was further assessed by polyacrylamide gel electrophoresis as shown in Fig.24. On resuspending the final pellet in cold MES buffer and sitting on ice for 1 h, the depolymerised protein (mainly tubulin) was assayed for colchicine-binding activity (For data see Chapter III, Table 3.4b). A typical purification scheme is shown in Fig.25.

#### 2.2.4 Precipitation with Vinca alkaloids

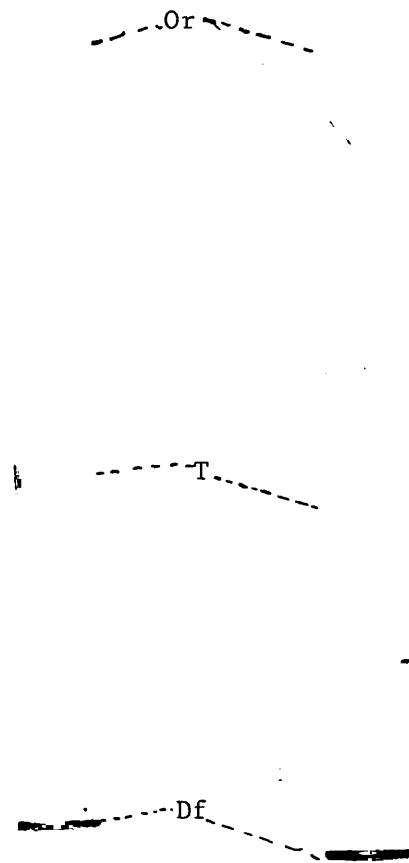
It was found that the addition of 500  $\mu$ M of vincristine (VC) or vinblastine (VB) induced the precipitation of 95-100 % of colchicine-binding activity present in either the initial high-speed supernatant fraction (IS), or in the redissolved second ammonium pellet (3P) from foetal rabbit brain (For data see Chapter III, 3.2.3. ). In these experiments, samples containing ca. 5 mg of protein/ml were incubated for 30 min at 37°C with 500  $\mu$ M (final concentration) of the Vinca alkaloid, during which the solution became faintly turbid. After cooling, the incubation mixtures were centrifuged at 100,000 g for 1 h at 4°C. The pellet and supernatant fractions thus obtained as well as the corresponding fractions from the control sample incubated without vincristine, were assayed for colchicine-binding activity by the DE 81 filter disc assay as described in section 2.3.4 and the protein in each fraction were analysed by polyacrylamide gel electrophoresis as shown in Fig.26. (For method see 2.5).



**Fig.2.6.** Electrophoresis pattern in SDS-polyacrylamide gels of high-speed foetal brain supernatant (supt) fraction and of derived vincristine-induced protein precipitate (Vc)

---

Supt.                      VC



Df = dye front

T = tubulin.

### 2.3 Determination of colchicine-binding activity

The specific binding of colchicine to tubulin provides a means for determining the amount of tubulin present in tissue extracts. Samples containing tubulin are first incubated with [ $^3\text{H}$ ]-colchicine, and the tubulin-bound colchicine complex is separated from free [ $^3\text{H}$ ] colchicine either by column chromatography on Sephadex G 100, or by selective adsorption of the tubulin-colchicine complex onto Sephadex DEAE A 50 or onto DE 81 filter disc (Whatman). Since the tubulin dimer (MW. 110,000) has been found to bind up to 1 mole of colchicine (Weisenberg et al., 1968), determination of the radioactivity in the sample can provide an indirect measure of the amount of tubulin, in as much as tubulin represents a single species of protein homogeneous with respect to its ability to bind colchicine.

#### 2.3.1 Incubation conditions

Colchicine-binding activity was routinely assayed by incubation of protein (up to 1 mg/ml incubation mixture) with 2.5  $\mu\text{M}$  (final)- [ $^3\text{H}$ ] colchicine in 0.6 ml (final vol) containing 10 mM-sodium phosphate- $\text{MgCl}_2$  buffer, pH 6.8 for 90 min at 37°C. Incubations were carried out in the dark to prevent any light-induced degradation of colchicine to luminocolchicine ( $\beta, \gamma$ ), a derivative which does not show high-affinity binding to tubulin (Wilson & Freidkin, 1966). The reaction was stopped by cooling on ice for 10 min and samples were taken for determination of protein-bound radioactivity by one of the methods outlined below.

#### 2.3.2 Column chromatographic assay using Sephadex G100

This assay was adapted from that described by Weisenberg et al., (1968). The whole procedure was carried out at 2-4°C. Sephadex G100 (about 10 g), was swollen overnight in distilled water and

Chromatographic separation of protein-bound and free <sup>3</sup>H colchicine on Sephadex G100 column.

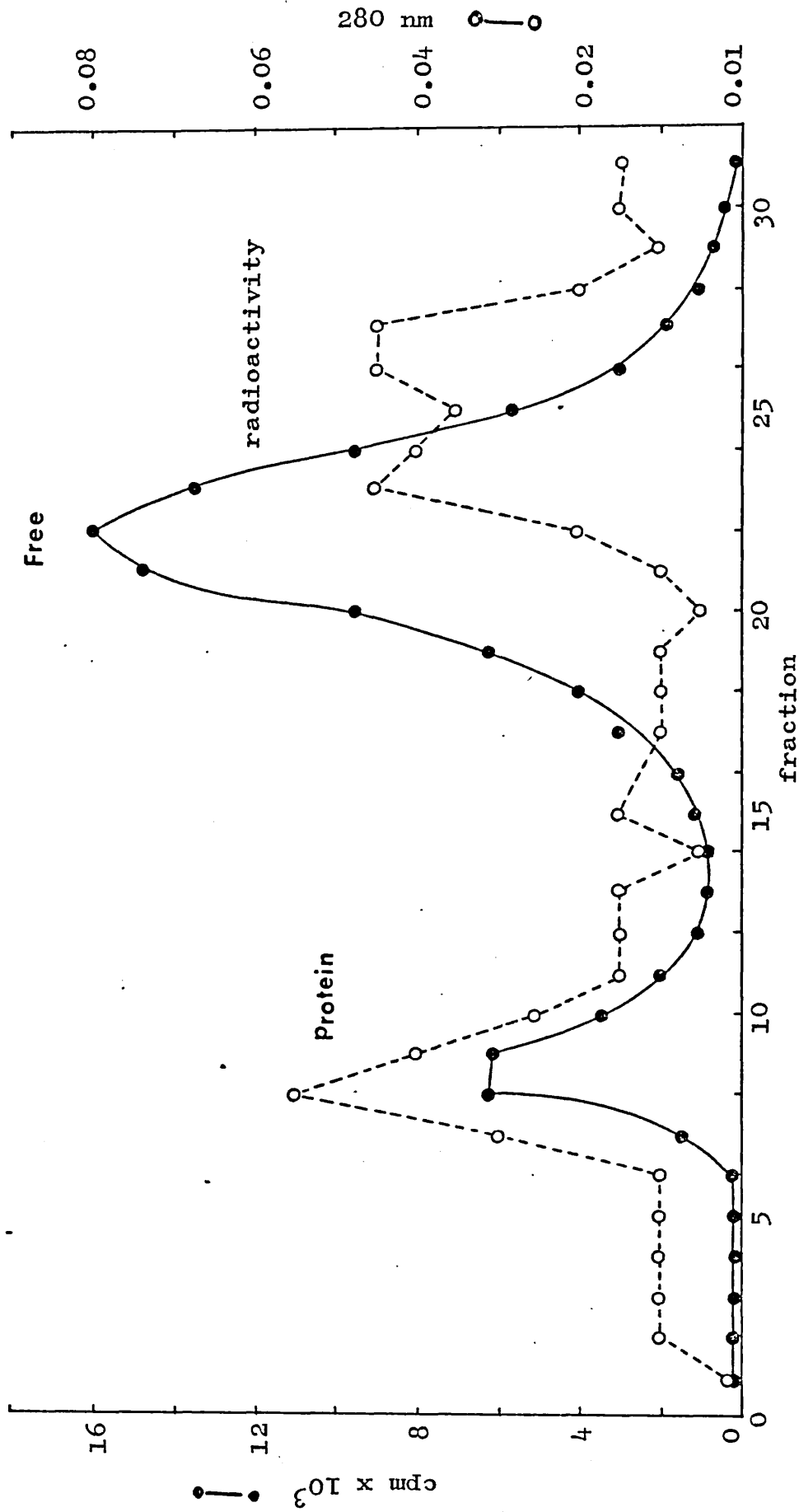


Fig.2.7.

Foetal rabbit brain tubulin incubated with <sup>3</sup>H colchicine was separated as specified in text, section 2.3.2.

equilibrated with P-Mg-GTP buffer. The Sephadex was then packed under gravity in 1 cm diameter glass columns to a height of 15 cm. Incubated samples (0.6 ml; see above) were loaded onto the columns, after being made dense with sucrose, and eluted with P-Mg-GTP buffer; 1 ml fractions were collected with a fraction collector. The protein content of each fraction was estimated by reading the absorbance at 280 nm in a Beckman spectrophotometer and aliquot of each fraction was taken for radioactive counting in 5 ml of Bray's (1951) scintillant, as described in section 2.8. A typical elution profile for tubulin purified by the batchwise method and incubated with [ $^3\text{H}$ ] colchicine as described above is shown in Fig.2.7. All assays were performed in duplicate.

### 2.3.3 Batchwise DEAE A50 Sephadex chromatographic assay (see Weisenberg, 1972)

Sephadex DEAE A50 (Pharmacia), after swelling overnight in distilled water, was treated with 0.5 N-HCl and 0.5 N-NaOH as recommended by the manufacturers, and finally equilibrated with P-Mg-GTP buffer. The reaction mixture incubated as described above (2.3.1) was added to 2 ml of packed Sephadex DEAE A50 in a conical centrifuge tube and stirred intermittently for 20 min on ice. The Sephadex which retains the protein-bound colchicine complex, was pelleted by centrifuging at 3,000 rpm for 15 min at 4°C and transferred directly into a vial containing 10 ml of Bray's scintillation fluid for radioactive counting. Assays of incubation samples were carried out in duplicate.

Table 21.

Comparison of colchicine-binding activity determined by different methods of assay

<u>Assay Method</u>	<u>Quenching Effect</u>	<u>Supernatant</u>	<u>Particulate</u>
		<u>Bound cpm x 10<sup>3</sup></u>	<u>Bound cpm x 10<sup>3</sup></u>
	<u>%</u>	<u>%</u>	<u>%</u>
1. Sephadex G100 column chromatography	6% ± 2	256 ± 3	73 ± 1
			100%
2. DEAE A50 Sephadex (Batchwise)	40% ± 5	236 ± 1	68 ± 2
			92%
3. DE 81 Filter Disc	8% ± 2	202 ± 10	48 ± 3
			79%
			66%

Legend to Table 21.

The results are expressed as total bound counts per ml of incubation mixture, after correction for quenching, which was determined in each case by an internal standardisation method. Values shown represent the means of duplicate determinations for 1 & 2 and means of triplicate determinations for 3 and the range of variation among determinations is given after each value. Percent values refer to the percentage of the value from assay method 1. Samples taken from the same incubation mixture for either supernatant or particulate fractions. Details of assays are as described in text.

#### 2.3.4 DE 81 filter disc assay (see Wilson, 1970; Lagnado et al. 1971)

In this method, Whatman DE81 filter discs (25 mm diameter) were numbered and placed on inverted plastic screw caps in petri dishes which were resting on ice. Aliquot of 0.1 ml from the incubation mixture was pipetted onto each filter disc which had been previously moistened with ice-cold 10 mM-Na phosphate buffer, pH 6.8. After allowing 10 min for absorption of protein-bound radioactivity, the discs were transferred to a beaker containing ice-cold 10 mM-Na phosphate buffer, pH 6.8 (30 ml per disc) and this washing medium 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 2.8). All assays were performed in triplicate (i.e. 3 discs for each incubated sample).

#### 2.3.5 Comparison of assay methods

The three methods of assay described above were compared in order to assess their reliability. In these experiments the high-speed supernatant and particulate fractions obtained from rat brain homogenates after centrifugation for 1 h at 100,000 g were used to assay colchicine-binding activity. Reaction mixtures containing 1.8 mg of protein per ml and 2.5  $\mu\text{M}$ -( $^3\text{H}$ ) colchicine in 10 mM-P-Mg buffer, pH 6.8 were incubated for 90 min at 37°C, and after stopping the reaction by cooling on ice for 10 min, 1 ml samples were taken for chromatography on Sephadex G100 (2.3.2); 0.6 ml samples were taken for the DEAE A50 Sephadex assay (2.3.3) and 0.1 ml samples for assay by the DE 81 filter disc method (2.3.4). The results obtained from these assays are summarised in Table 21.

In a separate experiment, the reliability of G100 Sephadex columns was examined by running two identical columns simultaneously. It was found that the results obtained were in agreement within less than 2 %. It is therefore, apparent that the results obtained by the methods using Sephadex G100 column chromatography or chromatography (batchwise) on DEAE A50 Sephadex gave a better recovery of protein-bound colchicine than was found using the DE 81 filter disc method. In view of the large numbers of samples which can be determined simultaneously by the filter disc method, using relatively small amount of sample; this method was chosen for routine assays of colchicine-binding activity.

#### 2.4 Determination of nucleotide-binding activity

Early measurements of the binding of tritiated nucleotides to tubulin were carried out by Sephadex G100 column chromatography as described above (For results see Chapter III, 3.2.4. ). The need to process large numbers of samples, however, necessitated the development of a more rapid and convenient method of assay, which will now be described.

##### 2.4.1 Incubation mixture

Reaction mixtures contained 100-200  $\mu\text{g}$  of protein in 0.3 ml of 10 mM Na-phosphate buffer (pH 6.8) containing 10 mM-MgCl<sub>2</sub>, and 6.5  $\mu\text{M}$  <sup>3</sup>H]GTP or <sup>3</sup>H]ATP (specific radioactivity ca. 1000 cpm/mole). Mixtures were incubated at 37°C for 15 min and reactions were stopped by dilution with 5 ml of ice-cold incubation buffer containing 0.1 mM of the appropriate nucleotide.

#### 2.4.2 Millipore filtration assay

The binding of tritiated nucleotide to protein was determined by a Millipore filtration assay adapted from the method of Tao *et al.* (1970). Incubated samples were filtered through Millipore filter discs (25 mm, HA 0.45  $\mu$ ) under mild suction, and the radioactivity retained on the discs was determined as described in section 2.8.2.

#### 2.5 Polyacrylamide gel electrophoresis

The purity and molecular weight of tubulin preparations were determined by polyacrylamide gel electrophoresis in three different buffer systems (see below), in the presence of sodium dodecyl (lauryl) sulphate (SDS; Fisons) used to dissociate proteins into their subunit polypeptides. Under these conditions, it is possible to calculate the apparent molecular weights of the individual polypeptides from their mobilities on basis of a standard curve obtained by plotting the logarithm of molecular weight versus the mobility for a series of monomer proteins of known molecular weight (see Weber & Osborn, 1969).

Electrophoresis was carried out on 7.5% or 10% acrylamide gel using three different buffer systems and details of the procedures employed will now be described.

In the phosphate buffer system, proteins were separated on 10% acrylamide gels prepared in 0.2 M-Na phosphate buffer, pH 7.0, containing 0.2% SDS, according to the method of Weber and Osborn (1969). Under these conditions, tubulin migrates as a single rather diffuse band (see Fig.1A). Bryan & Wilson (1971) discovered 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 conventions<sup>(Webb, 1964)</sup>. It was recently shown that purified tubulin can be separated into the same two components when electrophoresis is carried out in the presence of 2-8M-urea, using various buffer systems (Feit *et al.*, 1971; Wilson & Bryan, 1971; Fine, 1971; Lagnado *et al.*, 1972). Under these conditions it is not necessary to reduce, carboxymethylate and alkylate the protein to affect its resolution into  $\alpha$  and  $\beta$  monomers. In the present work the two forms of tubulin were separated in a discontinuous tris-glycerine buffered system modified from Laemmli (1970) in the presence of 4 M urea, according to the method described below (see Fig.1B).

In a few experiments, proteins were separated in a continuous tris-HCL buffered system according to the method of Walter and Matus (1974). This method, in which tubulin migrates as a single well-defined band, was mainly used in the analysis of the protein components of synaptic membrane preparations (see Chapter III, In this system, 7.5 % acrylamide gels were used, and the gel, electrophoresis and sample incubation buffers were adjusted to pH 7.4.

#### 2.5.1 General procedure for preparation of gels

Gels were prepared as described below, using a stock acrylamide solution containing 20 g purified acrylamide (Serva, Feinbio Chemica) and 0.15 g of NN'-methylene bisacrylamide (BDH) dissolved in 100 ml distilled water (for 10 % gels) or 15 g of acrylamide and 0.15 g of bisacrylamide dissolved in 100 ml of distilled water (for 7.5 % gels). To prepare 16 gels in 10 cm long siliconised glass tubes (internal

Table 2.2.Composition of electrophoresis and incubation buffers

	<u>Phosphate buffer system</u>	<u>Tris-glycine buffer system</u>	<u>Tris-HCL buffer system</u>
<u>Gel buffer</u>	0.2 M-Na-phosphate buffer, pH 7.0, 0.2% (w/v) SDS.	0.75 M-tris-HCL buffer, pH 8.8, 0.2% SDS. (w/v), 10 M-urea	0.2 M-tris-HCL buffer pH 7.4, 0.2% SDS (w/v)
<u>Electrophoresis Buffer</u>	0.1 M-Na-phosphate buffer, pH 7.0, 0.1% SDS	0.025 M-tris-0.192 M-glycine buffer, pH 8.3, 0.1% SDS (w/v).	0.1 M tris-HCL buffer pH 7.4, 0.1% SDS (w/v)
<u>Sample Incubation Buffer</u>	0.2 M-Na-phosphate buffer, 2% (w/v) SDS, 2% (v/v) $\beta$ -mercaptoethanol.	0.025 M-tris-HCL buffer, pH 6.8, 0.8% SDS (w/v), 2% $\beta$ -mercaptoethanol, 2 M-urea.	40 mM tris-HCL buffer pH 7.4, 0.04% SDS (w/v).

diameter 5 mm), 20 ml of appropriate gel buffer solution and 18 ml of the appropriate acrylamide solution were separately deaerated for 5 min and then mixed together with 2 ml of freshly prepared 1.5 % (w/v) aqueous solution of ammonium persulphate. Polymerisation was started by the addition of 0.04-0.06 ml of NNN'N'-tetramethylethylenediamine (TEMED, Koch-Light). The complete mixture was immediately poured into the glass tubes, stoppered at one end with Parafilm, and a drop of water was added to each tube to improve the meniscus of the gel. Gelling usually occurred within 20 min and the gels could be used within an hour of preparation.

#### 2.5.2 Buffer systems for electrophoresis

Three buffer systems, i.e. phosphate buffer, tris-glycine buffer and tris-HCL buffer, were used. The composition of the three different buffer systems are described in Table 22, together with the composition of the incubation buffer used to disaggregate protein samples prior to electrophoresis.

#### 2.5.3 Preparation of samples and electrophoresis

Protein samples were mixed with equal vol of appropriate incubation buffer (see Table 22) for 30 min at 50°C. After incubation and cooling, a drop of glycerol and bromophenol blue (saturated aqueous solution) were added to each incubated mixture and samples of 10-100  $\mu$ l, depending on protein concentration, were applied on the tube for electrophoresis, which was carried out until the tracking dye reached the end of the gel (about 6 h in phosphate buffer, 2-3 h in tris-glycine and tris-HCl buffer systems) using current of 6 mA/gel.

#### 2.5.4 Fixing , staining and destaining

Proteins in the gel were fixed in 10 % trichloroacetic acid (TCA) or sulphosalicylic acid (SSA) for at least one hour, and stained in a 0.25 % (w/v) solution of Coomassie Brilliant blue which was prepared by dissolving 1.25 g of the dye in 454ml of 50 % methanol and 46 ml of glacial acetic acid. Background staining of the gel was removed by washing with several changes of a destaining solution containing <sup>7%</sup> glacial acetic acid in 50 % methanol.

#### 2.5.5 Molecular weight determination

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

$$\text{Mobility} = \frac{\text{length before destaining}}{\text{length after destaining}} \times \frac{\text{distance migrated by protein}}{\text{distance migrated by dye}}$$

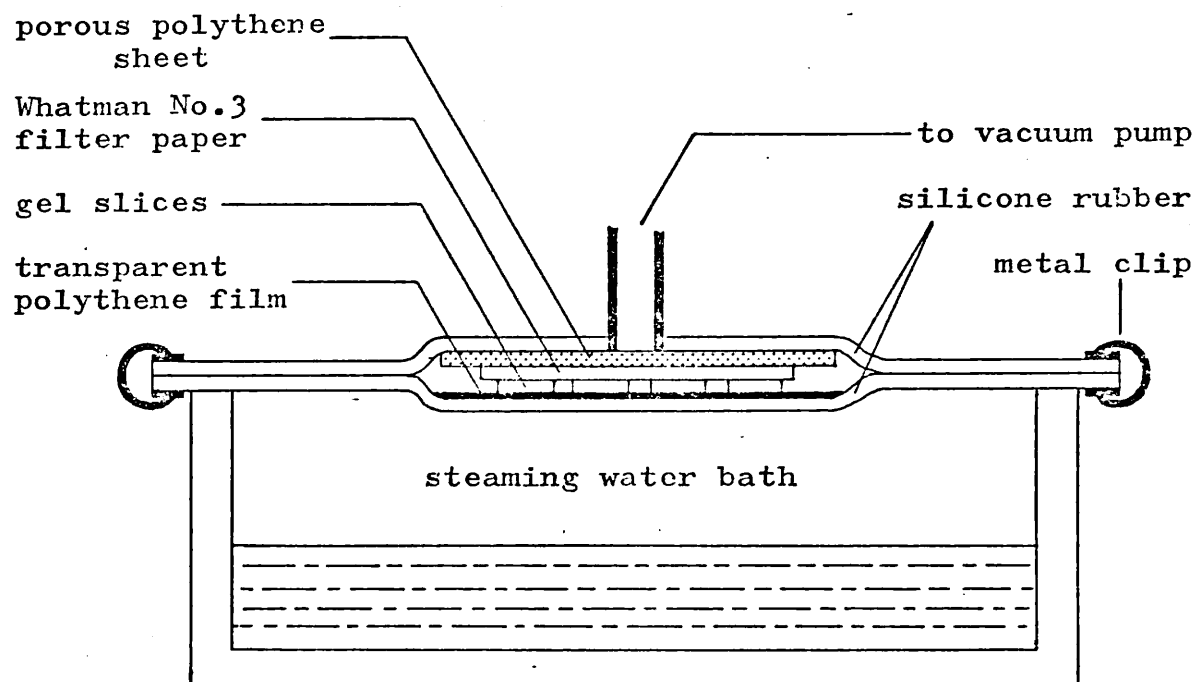
('gel factor')

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 (For data see Chapter III, Fig. 3.2.).

#### 2.5.6 Slicing and drying of gels for radioautography

Radioautographs were prepared by using air-dried longitudinal slices of stained gels by the methods similar to those originally described by Fairbanks et al. (1965). Gels were sliced in a home-made cutting apparatus and the inside slices (ca. 1.5 mm thick) were

Fig.2.8. Drying apparatus for polyacrylamide gel slices



dried onto Whatman No.3 filter paper under steam heating and reduced pressure. Drying was carried out in the apparatus as shown in Fig.2.8. The gel slices were placed on a transparent polythene sheet resting on a larger sheet of silicone rubber (TC 156, ESCO Rubber Ltd., London). They were then covered with a Whatman No. 3 filter paper, topped by a porous polythene sheet (50  $\mu$  size; Gallenkamp & Co. Ltd., U.K.) and silicone rubber sheet which contained a central outlet for evacuation. The silicone rubber sheets were sealed by metal clips and the whole device was placed on a steaming water bath; at the same time pressure was reduced by connecting the central outlet to a vacuum pump. The whole process took about 1 h and the dried gel slices stuck onto the filter paper were exposed to X-ray film (Ilford Red Seal 25 FW) for 2-4 days. This procedure was mainly used to detect protein bound [ $^{32}$ P] (see Chapter III, 3.4. ).

## 2.6 Thin layer chromatography

The purity of [ $^3$ H] colchicine, [ $^3$ H] GTP and [ $^3$ H] ATP were analysed by thin layer chromatography as described below.

### 2.6.1 [ $^3$ H] colchicine

Tritiated colchicine was chromatographed with authentic colchicine (50  $\mu$ g; Sigma) on 0.3 mm thick silica gel G (Kieselgel G; Merck) plates (20 x 20 cm). The plates were developed with methanol as<sup>o</sup> described by Wilson and Friedkin (1966). The dry plates were viewed under ultra-violet (UV) light to local the colchicine and any impurities. Usually, radioautogram of the dried plates revealed a single radioactive spot which corresponded precisely with the

authentic colchicine (Rf 0.56) detected under UV light.

Radioautography was carried out by exposing the plates to X-ray film for 3-4 weeks. Direct counting of the gel scraps ( $1 \text{ cm}^2$ ) along the colchicine spot showed that more than 95 % of radioactivity was associated with the authentic colchicine spot.

#### 2.6.2 Nucleotides

Thin-layer and paper chromatography were used to determine the purity of unlabelled or labelled nucleotides, and also for the identification of the species of nucleotide bound to tubulin preparations. Samples and authentic nucleotide standards ( $50 \mu\text{g}$  each) were separated by ascending chromatography on PEI (polygram CEL 300 PEI, Macherey-Nagel & Co., Duren, Germany) cellulose plates or on Whatman No.1 chromatographic paper, which were developed in solvents consisting of 1 M-LiCl/1 M-Acetic acid (1:1) (see Nazar, Lanford & Wong, 1970), or isobutyric acid/water/ammonia (0.88)/0.1 M-EDTA (100: 56: 4.2: 1.6 by vol) (Radiochemical Centre, solvent No. 73), respectively. The separated nucleotides were located under UV light and in case of radioactive nucleotides,  $1 \text{ cm}^2$  sections along the length of the chromatograms were cut and counted in 5 ml of Bray's scintillant. Alternatively, the radioactive purity of the nucleotide was qualitatively checked by radioautography after placing the chromatogram in contact with an X-ray film (Ilford Red Seal 25 FW) for about three weeks. Usually one radioactive spot was detected for labelled nucleotides, which accounted for 90-95 % of total counts applied after correction for quenching. Rf values for guanine and adenine nucleotides are shown below for the two separate systems.

Nucleotides chromatography

	<u>Rf value</u>					
	GTP	GDP	GMP	ATP	ADP	AMP
PEI-Cellulose Thin-layer plate	0.91	0.68	0.37			
Whatman No.1 Chromatographic Paper	0.76	0.72	0.625	0.54	0.47	0.40

2.7 Protein determination

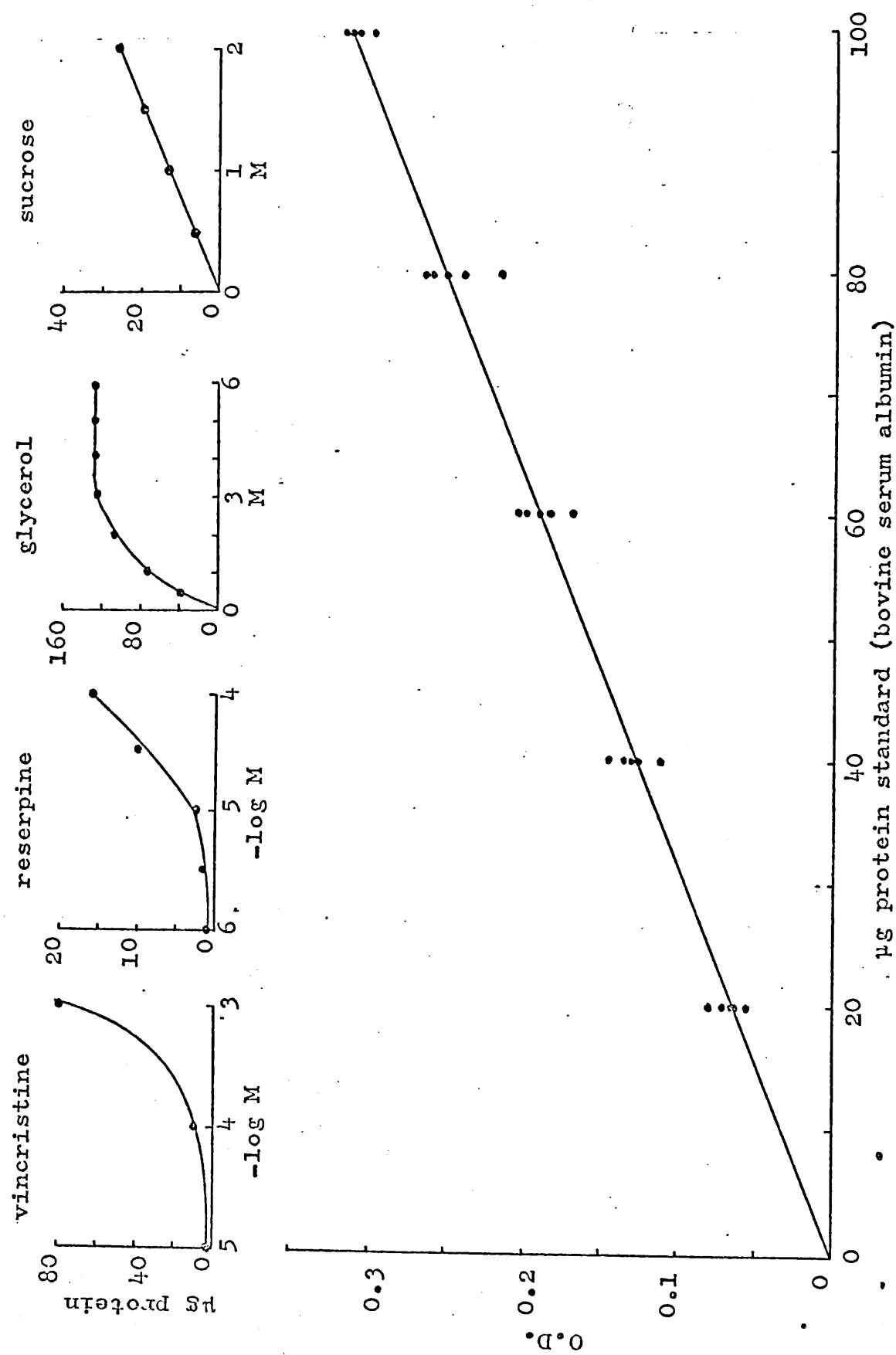
Proteins were determined according to the method of Lowry et al., (1951) by using the following solutions:

- A) 2%  $\text{Na}_2\text{CO}_3$  in 0.1 N-NaOH (20 g  $\text{Na}_2\text{CO}_3$ , anhyd. + 4.0 g NaOH/litre)
- B) 0.5%  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  in 1 % Na citrate.
- C) 50 parts of A freshly mixed with 1 part of B.
- D) Folin-Ciocalteu Reagent (as supplied by BDH) was diluted 1 : 2.3 with distilled water to give a solution which was 1 N with respect to acid.
- E) Protein standards were prepared from a stock solution containing 10 mg/100 ml of bovine serum albumin.

Samples containing up to 100  $\mu\text{g}$  of protein in a total vol of 0.8 ml were mixed with 4 ml of reagent C and allowed to stand for 10 min at room temperature. To this mixture, 0.4 ml of reagent D was added and mixed rapidly; the blue colour was allowed to develop for at least 20 min, before measuring the absorbance at 700 nm in a Beckman spectrophotometer against a blank without added protein. A typical standard curve based on 5 separate sets of data is shown in



Fig.2.9. Effects of various additives on protein estimation by Folin-Lowry method.



Insets show colour obtained in the absence of protein with various additives at concentrations indicated, expressed as equivalent µg of protein on the standard curve shown below for bovine serum albumen.

Fig.2.9, which shows that at 700 nm the optical density increases linearly with protein concentration between 0-100  $\mu\text{g}$  protein.

The effects of various compounds used in this work on the colour developed in the protein assay were tested. It is well known that indole compounds and glycerols affect the reaction by increasing the colour intensity. It was found that vincristine and vinblastine, being double indole compounds at a concentration of 1 mM (final concentration in 0.8 ml sample) gave an increase in optical density equivalent to 80  $\mu\text{g}$  of protein, but below 0.1 mM the effect was negligible. This is illustrated for vincristine in Fig.9a.

Reserpine, another indole alkaloid, gave an increase in colour corresponding to 16  $\mu\text{g}$  of protein when present at a concentration of 0.1 mM (see Fig.9b). Glycerol, at concentrations between 0.5 M and 3 M, increased the colour intensity in a linear fashion, giving values between 40 - 125  $\mu\text{g}$  of protein. Beyond 3 M, the effect of glycerol seemed to reach a plateau (see Fig.9c). At relatively high concentrations sucrose also reacted with the reagents to give a faint blue colour (Fig.9d). Other indole alkaloids such as yohimbine and harmine and other indole amines such as serotonin, tryptamine and melatonin gave a faint colour when present at a concentration above 10  $\mu\text{M}$ . It was therefore, necessary to include appropriate blanks when any of these compounds were present in the protein sample.

## 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.

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

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

It was found that the efficiency for  $\{^3\text{H}\}$  and  $\{^{32}\text{P}\}$  were about 48 % and 95 %, respectively.

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

#### 2.8.1 Preparation of radioactive solutions

Radioactive chemicals obtained from Radiochemical Centre were adjusted to the right concentrations with the same unlabelled compounds, and to contain sufficient radioactivity for the binding assays. Tritiated colchicine was usually obtained as 250  $\mu\text{Ci}$  in 0.25 ml of 50 % ethanolic solution containing specific radioactivities between 1.7 - 2.5 Ci/m mole. This solution gave a concentration of about 500  $\mu\text{M}$ , which was diluted with unlabelled colchicine, as shown below, to give a stock solution of final concentration 76  $\mu\text{M}$

	<u>Volume used</u>	<u>Final conc.</u>
Radioactive colchicine solution (500 $\mu\text{M}$ )	0.25 ml	20 $\mu\text{M}$
Unlabelled colchicine solution (1,400 $\mu\text{M}$ )	0.25 ml	56 $\mu\text{M}$
Water	5.75 ml	.
Total	<u>6.25 ml</u>	<u>76 <math>\mu\text{M}</math></u>

When 20  $\mu\text{l}$  of this stock solution equivalent to 0.4  $\mu\text{Ci}$  was added to incubation mixture (see 2.3.1) to make up a total volume of 0.6 ml, the colchicine was diluted 30 times to a final concentration of 2.5  $\mu\text{M}$ . The specific radioactivity in the mixture was approximately 530 cpm/mole at the efficiency of 48 %.

Similarly, the stock solutions of [ $^3\text{H}$ ] GTP (S.A. 12 Ci/mM, 250  $\mu\text{Ci}$  in 0.5 ml) and  $^3\text{H}$  ATP (S.A. 29 Ci/mM, 250  $\mu\text{Ci}/0.5$  ml) were prepared to give a concentration of 98.5  $\mu\text{M}$  and 95  $\mu\text{M}$  respectively. When 20  $\mu\text{l}$  of these solutions were used for incubation in a total volume of 0.3 ml, the final concentration of nucleotide was about 6.5  $\mu\text{M}$ . (see 2.4.1)

## 2.9 Expression of data and statistical analysis

Results of binding studies were expressed either in terms of number of radioactive counts per mg protein (cpm/mg protein) or, when purified tubulin was used, as moles of ligand bound per mole protein (MW 110,000). In some experiments, such as in the subcellular distribution studies, the results were also calculated in terms of relative specific activities (R.S.A.) as given:

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

This enabled better comparisons to be made amongst various fractions. Taking the R.S.A. of the original fraction as 1, values greater than 1 would indicate a relative enrichment of activity.

The means of duplicate or triplicate determinations in each experiment were obtained, and the means from separate experiments were pooled, averaged and analysed statistically to obtain their standard deviation (S.D.) or standard error (S.E.M) according to the

following formulae:

$$\text{S.D.} = \sqrt{\frac{n (\sum x^2) - (\sum x)^2}{n^2}}$$

$$\text{S.E.M.} = \frac{\text{S.D.}}{\sqrt{n}}$$

In certain cases, a Student's t-test was applied to calculate the level of significance.

Chapter III      Purification and binding properties of foetal  
rabbit brain microtubular protein

3.1      Introduction

3.2      Isolation and characterisation of foetal rabbit brain  
microtubular protein

3.2.1      Polyacrylamide gel electrophoresis

3.2.2      Colchicine-binding activity

3.2.3      Effects of Vinca alkaloids

3.2.4      Nucleotide-binding activity

3.2.5      Comparison of colchicine-, GTP- and ATP-binding activities

3.2.6      Specificity of nucleotide-binding sites

3.2.7      Effect of sucrose on colchicine- and GTP-binding activities

3.3      Comparison of the binding properties of tubulin and  
of soluble 'transfer factors' isolated from pig brain

3.3.1      Introduction

3.3.2      Results

3.4      Phosphorylation of tubulin in vitro and in situ

3.5      Phosphorylation of tubulin-like protein in synaptic  
membranes

3.6      Discussion

Chapter III      Purification and binding properties of foetal rabbit  
brain microtubular protein

3.1      Introduction

Preliminary investigation showed that soluble extracts from immature rat brain were relatively enriched in colchicine-binding protein as compared with extracts derived from adult brain. Further, in preliminary experiments using foetal and adult rabbit brain extracts obtained by centrifugation at 100,000 g for 1 h of a 10 % homogenate (see 2.1.2), it was found that the colchicine-binding specific activity (cpm/mg protein) of the crude supernatant and particulate fractions thus obtained from foetal brain was approximately twice that found for adult brain. For this reason, microtubular protein (tubulin) was purified from foetal rabbit brain, as a constant supply of this material was readily available. In this chapter, various methods of purification are compared and evidence that foetal rabbit brain is a rich source of tubulin is presented. The binding properties of the purified foetal rabbit brain tubulin were investigated. In the course of this investigation, a more convenient isotopic assay was developed for the determination of the nucleotide-binding activity of tubulin which was subsequently used to study the stability and specificity of nucleotide-binding sites. Additional experiments are also reported on the phosphorylation of tubulin in vitro and in situ. Finally, evidence is presented to show that synaptic plasma membranes isolated from pig cerebellum contain tubulin-like protein components.

### 3.2 Isolation and characterisation of foetal rabbit brain

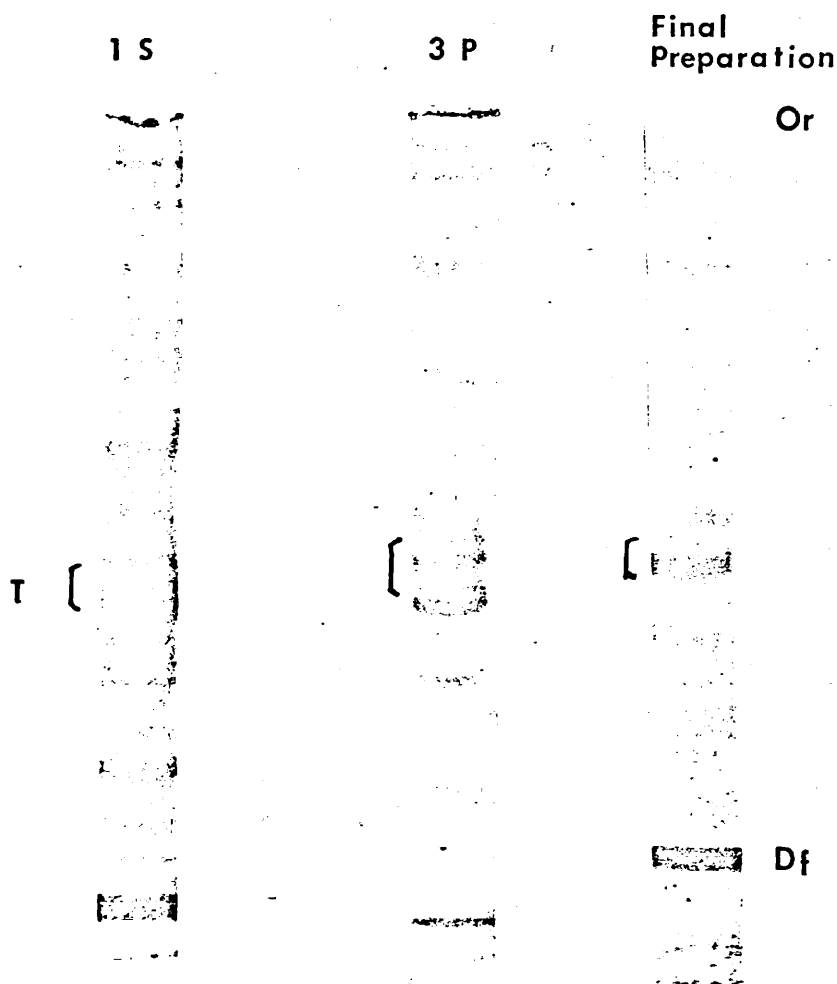
Foetal rabbit brain was used as a starting material for the isolation of tubulin which was subsequently characterised and used for the study of its biochemical properties. In this section, the results of different procedures used for the purification of tubulin from foetal rabbit brain will be discussed.

The chromatographic method (batchwise) of Karlsson & Sjostrand (1971), the gradient elution method as described by Bryan & Wilson (1971), and the polymerisation procedure (Shelanski *et al.*, 1973), were carried out as described in 2.2. , and the purification of tubulin was monitored in terms of enrichment in colchicine-binding activity and its behaviour during electrophoresis in SDS-polyacrylamide gels.

In the chromatographic method (batchwise method, see 2.2.1) adapted from Karlsson and Sjostrand (1971), it was found that the final tubulin preparation accounted for 2.5 % to 4 % of the total protein in the initial high-speed supernatant fraction (IS), and that the colchicine-binding activity per mg protein of the final preparation was increased by 4 to 10 times as compared to the activity of the initial supernatant. This relatively modest increase in specific activity during purification is partly attributed to the decay of colchicine-binding which occurred during the preparation (see Chapter V, 5.4. ). A considerable amount of tubulin was not precipitated at the 43 % ammonium sulphate step; it was found that by increasing the ammonium sulphate from 43 % to 60 % saturation at this step, an increase of 10 % - 15 % in the yield of tubulin was obtained in the final preparation. Comparison of the polyacrylamide gel electrophoresis



Fig. 3.1. Polyacrylamide gel electrophoresis patterns of fractions during purification of foetal rabbit brain tubulin by batchwise chromatographic method.



Fractionation of high-speed brain supernatant was carried out as shown in Section 2.2.1 and Fig.2.2.

Electrophoresis : in tris-glycine urea system, as indicated in Section 2.5.2.

1 S : initial high-speed supernatant fraction

3 P : 2<sup>nd</sup> ammonium sulphate precipitate

Final preparation : 0.8 M KCl eluate from DEAE-A50 Sephadex

T = tubulin

Df = dye front

Or = origin

(Note that in this experiment, tubulin of final preparation was not resolved into  $\alpha$  and  $\beta$  forms)

patterns of the various fractions obtained in the purification procedure clearly shows a progressive enrichment of tubulin (see Fig.3.1). This method yields approximately 1 mg of tubulin per g fresh weight of tissue.

In the gradient elution method (2.2.2), the yield of tubulin was higher, (about 11 % of the total protein in the high-speed supernatant (1S)), but the colchicine-binding activity per mg of protein was increased by only 2 to 3-fold. This might be explained by the fact that tubulin was in contact with high concentrations of NaCl throughout the period of elution, and that this high salt concentration might rapidly inactivate the colchicine-binding activity of tubulin as has been suggested by Weisenberg et al. (1968). Indeed, it was noticed that in the presence of high concentration of NaCl (0.8 M) inactive tubulin aggregates were formed in the samples during storage at 4°C before assay. The purity of tubulin obtained by this method is comparable to that purified by the batchwise method. Although this method is a simple one-step procedure, the tubulin obtained is rather inactive in terms of its colchicine-binding activity.

The precipitation of tubulin from the initial high-speed supernatant (1S) by the addition of Vinca alkaloids (see Marantz & Shelanski, 1970; Weisenberg & Timasheff, 1969; Wilson et al., 1970; Lagnado et al., 1971) can be used to obtain partially purified tubulin. However, under these conditions various other proteins are also precipitated together with tubulin(see 2.2.4.).

Tubulin purified by the microtubule assembly -disassembly procedure of Shelanski et al.(1973) (see also Chapter II, 2.2.3) usually bound twice as much colchicine as that purified by the

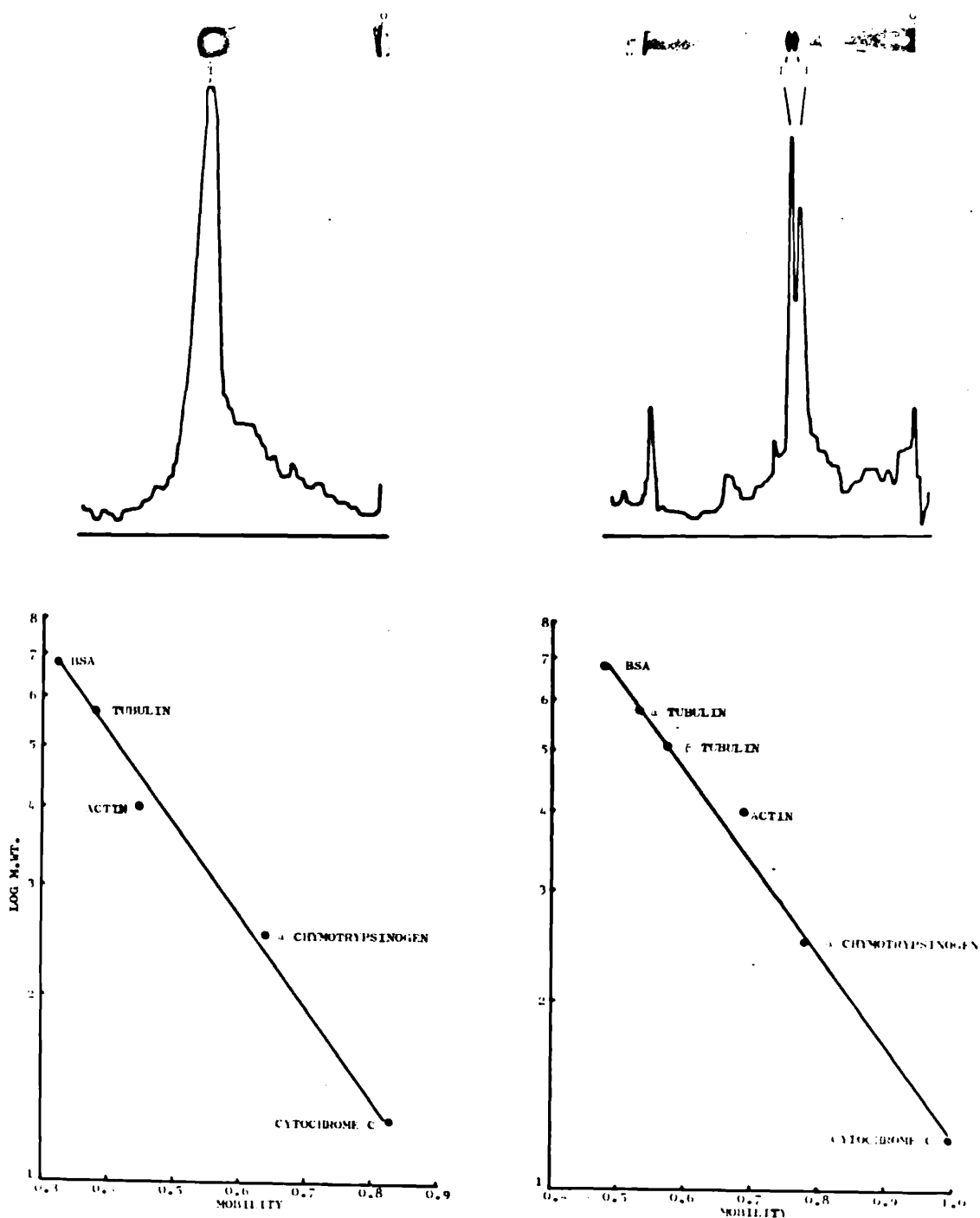
batchwise method. The final preparation accounted ~~for~~ 3 % to 4 % of the total protein in the initial high-speed supernatant fraction (1S). Electron microscopic examination of the polymerised microtubules showed an abundance of characteristic cylindrical structures 25 nm in diameter (see Chapter VI, Fig. 6.2). When a sample of assembled microtubules was depolymerised by cooling on ice, and analysed by electrophoresis on polyacrylamide gels in both phosphate buffered and tris-glycine buffered systems (Chapter II, 2.5.2), the molecular weight as determined by relative mobility (see section 3.2.1 & Fig. 3.2) was consistent with the values obtained from other preparations by different methods. In addition to the  $\alpha$  and  $\beta$  tubulin subunits obtained in the tris-glycine buffered system, or a single band in the phosphate-buffered system, 2 - 3 high molecular weight bands near the origin of the gel were also observed. However, the actin-like band (M.W. 36-45,000) which was usually detected on samples purified by other methods was absent in preparations obtained by this method.

### 3.2.1 Polyacrylamide gel electrophoresis

Both the purity of tubulin and the molecular weight of its subunits were determined by polyacrylamide gel electrophoresis.

Purified tubulin gave one prominent band in the phosphate buffered system as shown in Fig. 3.2 irrespective of the method of purification. The molecular weight of tubulin in this system was found to be  $56,000 \pm 3,000$  (s.d.; n=4). Very little contamination was seen as judged by the densitometric scan of the gel (Fig. 3.2). Occasionally, 2 - 4 minor protein bands of

Fig. 3.2. Molecular weight determination of fetal rabbit brain tubulin purified by the batchwise chromatographic method



Protein was purified as indicated in section 2.2.1. & Fig.2.2. Electrophoresis was carried out on SDS-polyacrylamide gels in a phosphate buffered system (a) or in a tris-glycine-urea buffered system (b).

Top : stained gels & corresponding densitometric scans

Bottom: standard curves for estimating molecular weight on the basis of electrophoretic mobility (see section 2.5.5. and text, section 3.2.1.)

high molecular weight were present close to the origin; this was especially evident in the tubulin preparations isolated from in vitro reassembled microtubules. In the tris-glycine buffered system, tubulin gave two closely related bands (Fig.3.2) and in keeping with the IUPAC conventions for the naming of protomers in multisubunit proteins (Webb, 1964), the faster-moving band will be referred to as the  $\beta$  tubulin subunit, and the slower-moving band as the  $\alpha$  tubulin subunit (see also Bryan & Wilson, 1971). The molecular weight of  $\beta$  and  $\alpha$  subunits were found to be  $52,000 \pm 2,000$  and  $58,000 \pm 2,000$  ( $n=3$ ), respectively (see Fig.3.2).

At times there was difficulty in reproducing the doublet pattern of tubulin in this system. It was found, however, that when the incubation buffer was diluted to one fifth its strength (i.e 25-mM tris-HCl buffer pH 6.8, containing 0.8 % SDS and 2 % -mercaptoethanol) in order to adjust the weight ratio of SDS to protein to between 1.5 : 1 and 2 : 1, the doublet pattern was reproducibly obtained. The reasons for this behaviour are not clear, although it has been observed for other proteins that excess concentrations of SDS in relation to the amount of protein used give rise to anomalous behaviour during polyacrylamide electrophoresis. Bryan (1974) has recently shown that the separation of tubulin into two subunits during electrophoresis under similar conditions can also vary as a function of the ionic strength and pH of the buffer system; the effects of pH and ionic strength were taken to indicate a charge difference between the  $\alpha$  and  $\beta$  subunits. On the other hand, Bibring and Baxandall (1974) showed that the separation of the two subunits is also dependent on the amount of protein loaded on the gel. These observations will be discussed further in the final chapter.

Table 3.1.

The distribution of protein and colchicine-binding (CB) activity in a typical purification of foetal rabbit brain tubulin by the batchwise method.

(For outline of purification scheme, see Chapter II, 2.2.1 and Fig.2.2)

<u>Fraction</u>	<u>Total protein recovered/ fraction (mg)</u>	<u>Total CB activity/ recovered/ fraction (cpm x 10<sup>3</sup>)</u>	<u>Specific Activity (cpm x 10<sup>3</sup>/ mg of protein)</u>
Homogenate (H)	480	4766	9.93
1 <sup>st</sup> Supernatant (1S)	145	5044	34.79
1 <sup>st</sup> Particulate (1P)	270	1823	6.75
1 <sup>st</sup> (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> Supernatant (2S)	146	19826	135.8
1 <sup>st</sup> (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> Precipitate (2P)	11	1342	122.0
2 <sup>nd</sup> (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> Supernatant (3S)	86.4	15725	182.0
2 <sup>nd</sup> (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> Precipitate (3P)	36.95	7242	196.0
DEAE Sephadex break-through fraction	5.28	28	5.37
0.4M NaCl Eluate	6.48	39	5.94
0.8M NaCl Eluate* (dialysed)	10.2	2462.2	241.4
Yield as % of 1S fraction	7 %	49 %	7 x (Purification factor)

\* Final tubulin preparation used to assay CB-activity.

Table 3.2.

The distribution of protein and colchicine-binding (CB) activity in a typical purification of tubulin by the assembly of microtubules in vitro from foetal rabbit brain

(for outline of purification scheme, see Chapter II, 2.2.3 & Fig. 2.5).

<u>Fraction</u>	<u>Total protein recovered/ fraction (mg)</u>	<u>Total CB activity recovered/ fraction (cpm x 10<sup>3</sup>)</u>	<u>Specific Activity (cpm x 10<sup>3</sup>/mg protein)</u>
Homogenate (H)	599	14,316	23.9
1 <sup>st</sup> Supernatant (S1)	180	10,584	58.8
1 <sup>st</sup> Particulate (P1)	419	14,584	34.5
2 <sup>nd</sup> Supernatant (S2)	162	12,960	80.0
2 <sup>nd</sup> Particulate (P2)	34.2	15,236	445.5
3 <sup>rd</sup> Supernatant (S3)	13.92	6,011	431.8
3 <sup>rd</sup> Particulate (P3)	11.2	3,270	292.0
4 <sup>th</sup> Supernatant (S4)	8.1	3,622	447.2
4 <sup>th</sup> Particulate (P4)*	6.4	3,456	540.3
<hr/>			
Yield as % of			
SI fraction	3.5 %	32.6 %	9.2 x (Purification factor)

\* Final preparation of microtubules which was resuspended in M.E.S. buffer (1 - 2 mg protein / ml) and stored at 4°C (30 - 60 min) to depolymerise microtubules for assaying CB - activity.

### 3.2.2 Colchicine-binding activity of tubulin

Throughout the various purification procedures, the colchicine-binding activity of each fraction was assayed by the filter disc method so as to monitor the distribution of tubulin. In both the batchwise method and the method of microtubule assembly in vitro, a gradual increase in colchicine-binding activity was detected after each step of purification, although in the batchwise procedure, the colchicine-binding activity of the ammonium sulphate treated fractions (i.e. 2S, 2P, 3S & 3P) seemed to give much higher values than expected (see Table 3.1 & 3.2). Since all the fractions were assayed at the same time at the end of the purification procedure, on the day of preparation, it is possible that the high activity found in the fractions containing ammonium sulphate was due to a stabilisation of colchicine-binding activity by this salt. However, when incubation mixtures containing purified tubulin were brought to 32 % or 60 % saturation with ammonium sulphate, no obvious effect was observed on the binding activity. (Data <sup>for</sup> both control and test <sup>gave</sup> values, in  $\text{cpm} \times 10^3/\text{mg}$ , of  $132 \pm 3$ , and  $140 \pm 5$  ( $n = 3$ ), respectively). The yield of purified tubulin by the batchwise method was between 2.5 - 4 %, and the specific activity increased by 4 - 10 times. The amount of bound colchicine was estimated to be  $0.04 \pm 0.02$  mole/mole of tubulin dimer ( $n = 7$ ), taking a molecular weight of 120,000 for the tubulin dimer. This value is rather low in comparison to the values of 0.6 - 1.0 mole/mole of dimer determined for adult pig brain tubulin isolated by the same method of Weisenberg et al. (1968). This discrepancy is probably due to the omission of GTP <sup>during</sup> purification (see 3.3.1), since under



these conditions, there is a marked aging of colchicine-binding activity, as was first noted by Weisenberg et al. (1968). On the other hand, foetal rabbit brain tubulin purified by the method of microtubule assembly in stabilising medium containing added GTP (see 2.2.3), which resulted in almost the same yield of tubulin as in the batchwise method, with  $0.5 \pm 0.05$  (s.d.;  $n = 4$ ) mole of colchicine/mole of protein dimer (see table 3.4b.). Thus, we are not dealing with a species difference in the colchicine-binding of purified tubulin.

### 3.2.3 Effect of Vinca alkaloids

Several reports are available that vinblastine induces the aggregation of tubulin in vitro (Weisenberg & Timasheff, 1969; Wilson et al., 1970). The same effect was found with vincristine (Lagnada et al., 1971). This was investigated further on tubulin isolated from two sources of tissue, foetal rabbit and adult rat brain. The tissues were homogenised in 10 mM-P-GTP buffer, pH 6.8 containing 0.24 M-sucrose with or without the addition of 0.5 mM (final concentration) vincristine. Homogenates were centrifuged at 100,000 g for 1 h and the resulting supernatants and reconstituted pellets were assayed for colchicine-binding activity by the Filter Disc method. In the samples which had not been treated with vincristine, the supernatant fractions were enriched in colchicine-binding activity as compared with the particulate fractions (see Table 3.3). In the vincristine-treated samples, the reverse occurred, and the particulate fractions were found to account for more than 90 % of the recovered

Table 3.3.

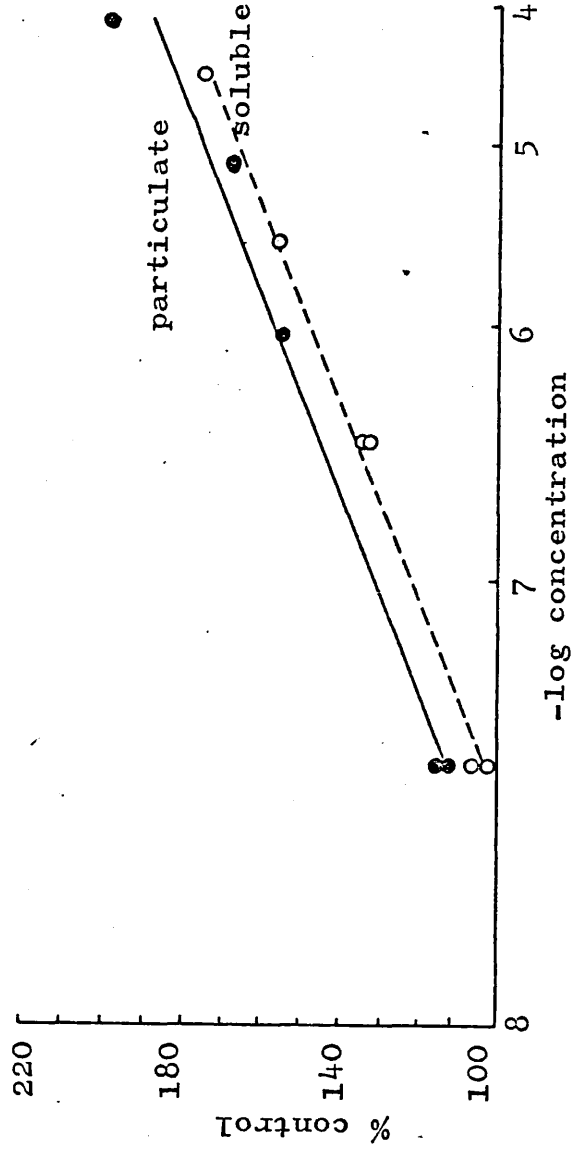
The effects of vincristine (Vc) on the distribution of colchicine-binding (CB) activity

	<u>Foetal rabbit brain homogenate</u>			<u>Adult rat brain homogenate</u>		
	<u>Control</u>	<u>*S</u>	<u>*P</u>	<u>Vc-treated</u>	<u>S</u>	<u>P</u>
% of protein recovered	31.7	68.3	25.6	74.4	22.9	77.1
% of counts recovered	44.6	55.4	5.4	94.6	45.5	54.5
Relative specific activity	1.4	0.81	0.21	1.27	1.99	0.71
					0.27	1.2
					21.2	78.8
					5.8	94.2
					0.27	1.2

Tissues were homogenised in the presence of added Vc and (0.5 mM, final), supernatant (\*S) and particulate (\*P) fractions were obtained by centrifugation of the homogenates (H). The three fractions (H, S & P) were assayed for CB-activity. For details see section 3.2.3.

Fig 3.3.

Effects of various concentrations of vincristine on the colchicine-binding (CB) activity of the soluble and particulate fractions obtained from foetal rabbit brain homogenates



In these experiments, the drug was added at the concentrations indicated to the assay mixtures used to determine CB activity. Data are given as percentage of CB activity determined in the absence of added drug (i.e. control = 100 %).

colchicine-binding activity. These data show that vincristine, like vinblastine (0.5 mM), also induces aggregation of tubulin which can be precipitated along with other proteins by high-speed centrifugation. Furthermore, the addition of vincristine (0.5 mM, final concentration) to high-speed supernatant from untreated homogenate also led to the selective precipitation of more than 90 % of the soluble colchicine-binding activity (data not shown), and this procedure can be used to obtain partially purified tubulin from soluble brain extracts. Further investigation showed that vincristine increased the colchicine-binding activity of the soluble and particulate fractions of brain in a concentration-dependent manner between 0.1  $\mu$ M to 0.1 mM vincristine (Fig.3.3). More direct studies on the binding of [ $^3$ H]-vincristine to tubulin will be reported in the next chapter. The effects of Vinca alkaloids on the binding properties of tubulin will be discussed further in Chapter V.

#### 3.2.4 Nucleotide-binding activity of tubulin

It has been shown that tubulin purified from pig brain contains two binding sites for guanine nucleotides, one of which is readily exchangeable (Weisenberg et al., 1968; Berry & Shelanski, 1972). However, very little is known about the function of these bound nucleotides. Therefore, experiments were carried out to study the properties of these nucleotide-binding sites. In preliminary experiments, the binding of [ $^3$ H]-GTP to purified tubulin was determined by Sephadex G100 column chromatography (for details of methods see Chapter II, 2.3.2). Under the same conditions, it was found that [ $^3$ H]-ATP was also bound to tubulin,

Legends to Fig. 3.4 and 3.5

Chromatographic separation of free and tubulin-bound  $^3\text{H}$  labelled nucleotides ( Fig. 3.4 :  $^3\text{H}$  GTP ; Fig.3.5:  $^3\text{H}$  ATP) on Sephadex G100 columns.

---

Chromatographically purified tubulin from foetal rabbit brain was incubated separately with either  $^3\text{H}$  - colchicine,  $^3\text{H}$  GTP or  $^3\text{H}$  ATP, and each incubated sample was chromatographed individually on a column of Sephadex G100. Experimental procedures used are as described in text, section 3.2.4. The same batch of tubulin was used each experiment.

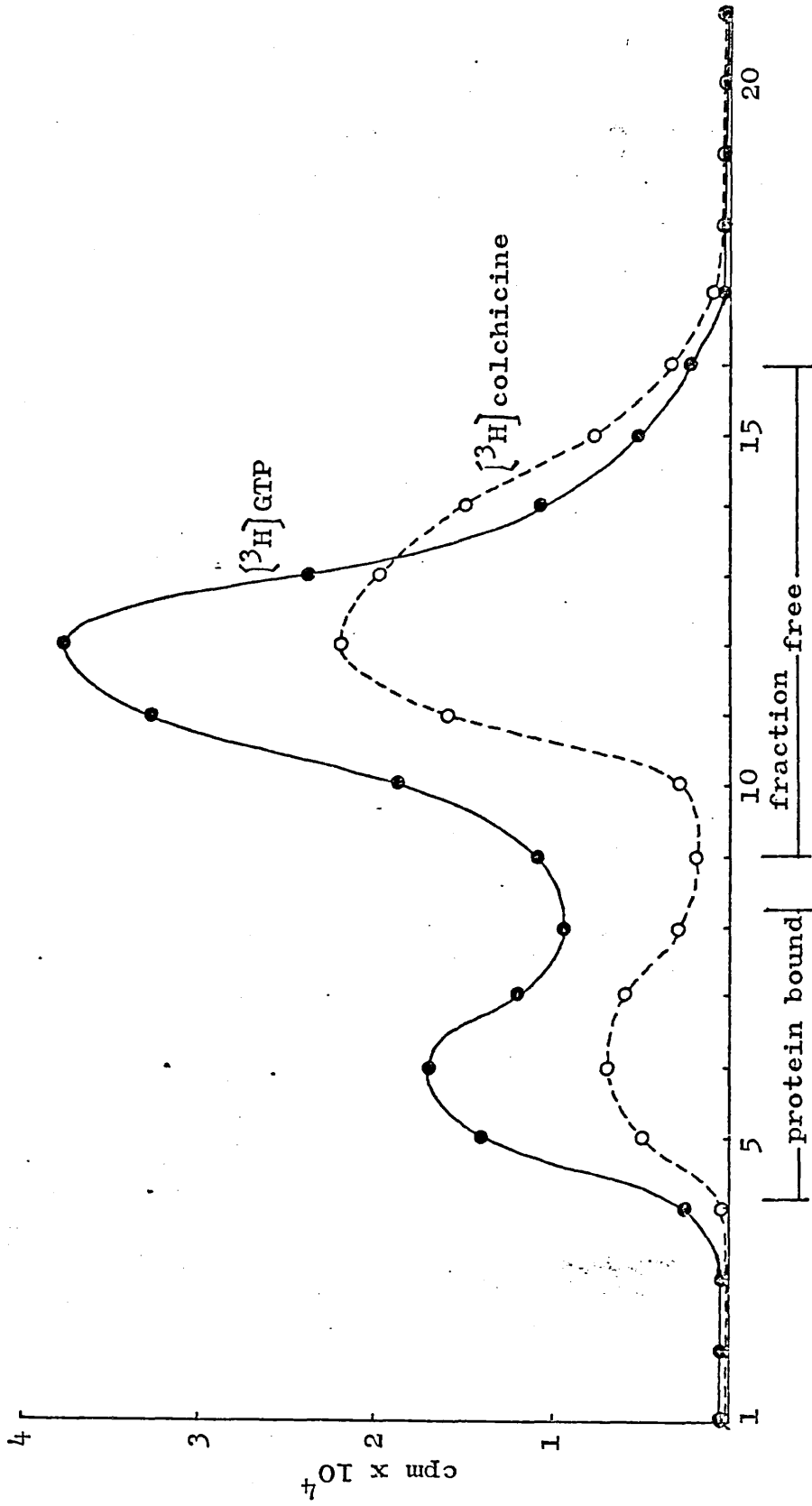
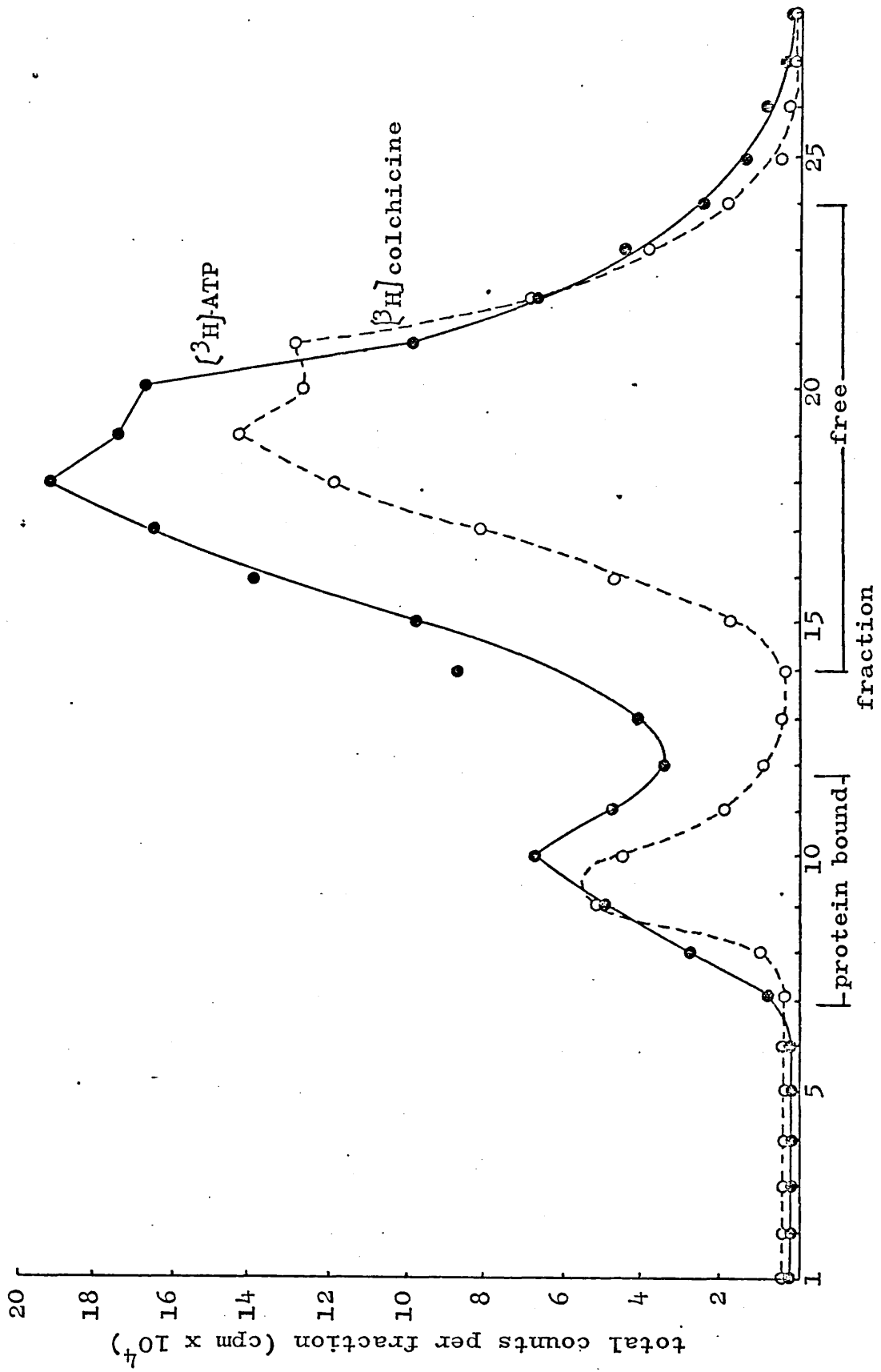


Fig.3.4.

Fig.3.5.



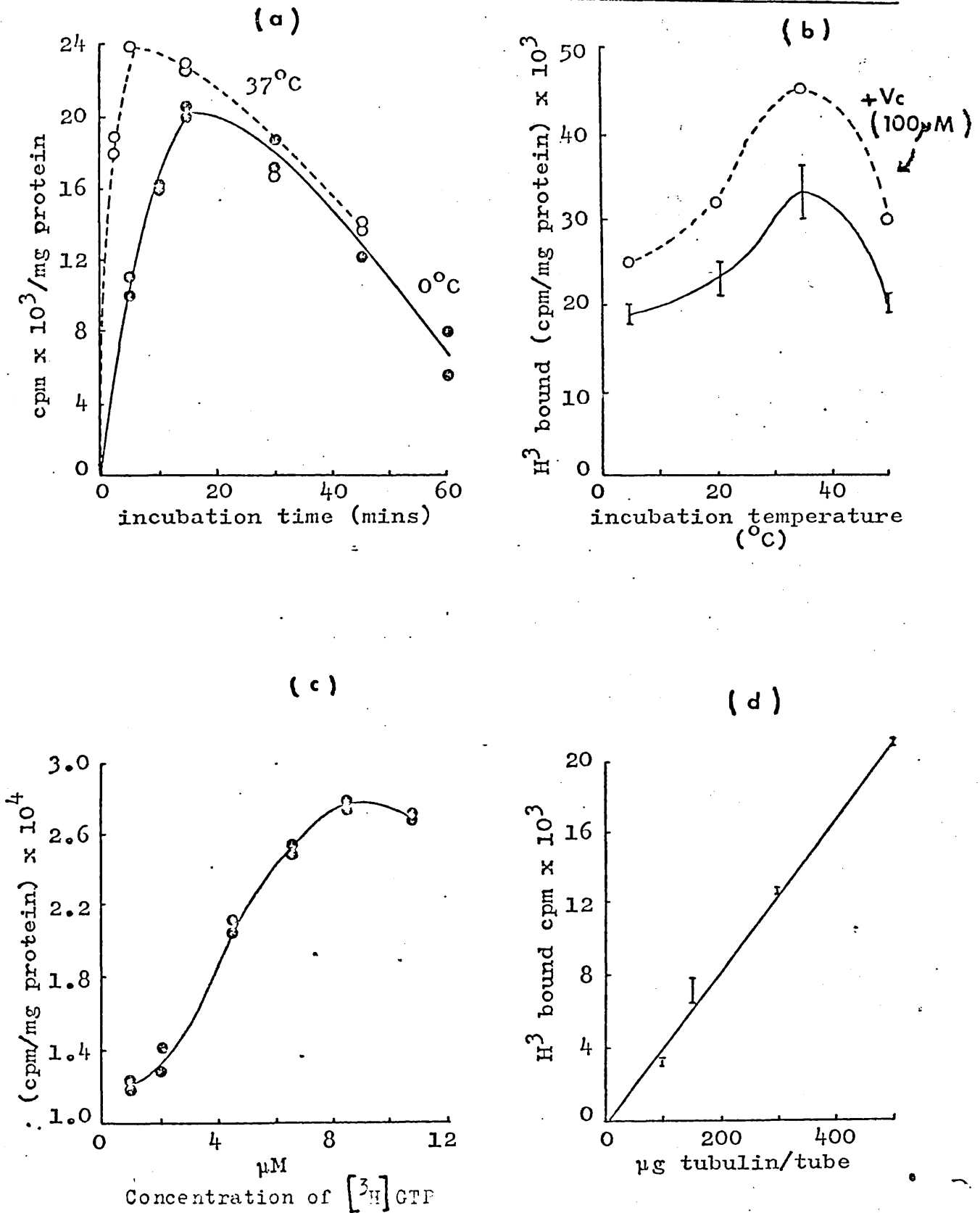
since both the tubulin-bound- $^3\text{H}$ -GTP and tubulin-bound- $^3\text{H}$ -ATP peaks coincided with the tubulin-bound- $^3\text{H}$ -colchicine peak (see Fig.3.4 & 3.5).

In order to extend this study, a more rapid assay method was desirable. The DE 81 filter disc assay for colchicine-binding activity was found to be unsuitable due to the retention by discs of both free and bound nucleotides. A millipore filtration assay, which was used by Tao *et al.*, (1970) in the study of cyclic AMP binding, was adapted for measuring the nucleotide-binding activity of tubulin (for details see Chapter II, 2.4.2). The reproducibility and reliability of this assay were determined by comparing the data with those obtained from the Sephadex G100 column chromatography. It was found that the Millipore filtration assay was 60 - 80 % as efficient as the chromatographic method. The difference between duplicates in the same experiment was usually found to be within 15 %. Heat-denatured tubulin (5 min at 100°C) and bovine serum albumin, used as blanks in the assay, gave an insignificant retention of radioactivity, less than 10 % of that of the active tubulin.

The basic conditions for measuring nucleotide-binding activity using the Millipore filtration assay were determined by using foetal rabbit brain tubulin prepared by the batchwise method. In earlier experiments, it was found that when  $^3\text{H}$ -GTP was added at a final concentration of 6.5  $\mu\text{M}$ , maximum binding at 37°C occurred within the first 5 min of incubation, and the level of bound radioactivity remained fairly constant for at least 15 min. When assays were conducted under the same conditions but at 4°C, maximum binding occurred between 15 to 30



Fig.3.6. The binding of [ $^3\text{H}$ ]GTP to chromatographically purified foetal rabbit brain tubulin



Incubations conditions as indicated in text, Section 3.2.4., and in chapter II, section 2.4.1.

Samples containing 100-200  $\mu\text{g}$  tubulin (a, b, & c) were incubated in the presence of 6.5  $\mu\text{M}$  [ $^3\text{H}$ ]GTP (in a, b, & d), for 15 min at 37 $^{\circ}$  (in b, c, & d). Other conditions as specified in Figures.

Vc = vincristine (b).

min (Fig.3.6a). Furthermore, when assays were conducted at various temperatures (for 15 min), highest binding activity was found to occur at 37°C; the activity measured during incubation at 4°C was about 60 % of that found at 37°C (Fig.3.6b). At temperatures above 50°C, rapid inactivation of GTP-binding activity occurred, and no activity could be detected at temperatures above 60°C. An interesting observation made in the course of these experiments was that vincristine (100  $\mu$ M, final) increased GTP-binding activity and to about the same extent, at all temperatures tested (Fig.3.6b, top curve). This apparent stimulation of GTP-binding by vincristine was further investigated (for results see Chapter V)

It was found that on increasing the concentration of GTP in the incubation mixture, maximum binding activity was attained at a concentration of about 8  $\mu$ M (see Fig.3.6c). Nevertheless, for most experiments reported, a concentration of 6.5 $\mu$ M-GTP was employed in the binding assays, since preliminary experiments had erroneously indicated that this concentration gave maximal binding).

Finally, it was shown that there was a linear relationship between the bound radioactive and protein concentration at concentrations of tubulin ranging between 100 and 500  $\mu$ g protein /0.3 ml incubation mixture (Fig.3.6d)

### 3.2.5 Comparison of the GTP-, ATP- and colchicine-binding activity of tubulin

The ATP-binding activity of tubulin preparations was tested under the same conditions of assay as those used to measure GTP-

Table 3.4

(a) Colchicine and nucleotide binding activity of tubulin purified from 28-day foetal rabbit brain by the batchwise chromatographic procedure

$[^3\text{H}]$ Colchicine	$[^3\text{H}]$ GTP	$[^3\text{H}]$ ATP	$\frac{\text{GTP}}{\text{ATP}}$ Ratio
	p moles bound / mg protein		
$320 \pm 68$ (n = 7)	$427 \pm 130$ (n = 17)	$253 \pm 130$ (n = 4)	$1.7 \pm 0.6$
$0.05 \pm 0.01$	moles bound / $1.2 \times 10^5$ g protein		
	$0.04 \pm 0.015$	$0.03 \pm 0.015$	

(a) Tubulin (200-400  $\mu$ g/ml incubation mixture) was isolated from foetal rabbit brain in the absence of added GTP by the batchwise chromatographic method (see Chapter II, 2.2.1).

(b) Colchicine and nucleotide binding activities of microtubular protein purified by assembly in vitro from guinea-pig cerebral cortex

<u>Expt.</u>	$[^3\text{H}]$ Colch.	$[^3\text{H}]$ GTP	$[^3\text{H}]$ ATP	$\frac{\text{GTP}}{\text{ATP}}$ Ratio
		<u>pmoles bound / mg protein</u>		
1.	5173	800	65	12.4
2.	5149	3765	246	15.3
3.	4543	1395	123	11.3
(3*)	(1089)	( 487)	(140)	( 3.5)

moles bound /  $1.2 \times 10^5$  g protein

<u>Mean</u>			
(1-3)	0.59	0.238	0.017
S.D.	0.04	0.14	0.006

\* Preparation 3 aged 18 h at  $-10^\circ\text{C}$  before assay.

(b) Tubulin ( $400 - 500$ )  $\mu\text{g/ml}$  incubation mixture) was purified from guinea-pig cerebral cortex by the microtubule assembly procedure (see Chapter II, 2.2.3).

In both sets of experiments, colchicine-binding activity was assayed by DE 81 filter disc method (Chapter II, 2.3.4) after incubation of the protein for 90 min at  $37^\circ\text{C}$  in  $10 \text{ mM}$ -phosphate;  $10 \text{ mM}$ -Mg-Cl<sub>2</sub> buffer, pH 6.8, containing  $2.5 \mu\text{M}$   $[^3\text{H}]$ colchicine. Nucleotide-binding data are based on the Millipore filtration assay (Chapter II, 2.4) after incubating the protein for 15 min at  $37^\circ\text{C}$  in the same buffer, containing  $6.5 \mu\text{M}$   $[^3\text{H}]$  nucleotide (S.A.  $10^3$  cpm/ pmole).

binding activity, i.e. incubation at 37°C for 15 min in the presence of 6.5  $\mu$ M [<sup>3</sup>H]-ATP. The tubulin used in these experiments was purified from foetal rabbit brain by the batchwise method, but in the absence of added GTP, since the omission of GTP during preparation was found to increase GTP-binding of the purified protein by about two-fold (see <sup>Fig.</sup> 3.10a). Under these conditions, the amount of ATP bound to tubulin was found to be three-quarters the value found with GTP. Taking the molecular weight of tubulin to be 120,000, the amounts of colchicine, GTP and ATP bound per mole of tubulin dimer gave mean values of 0.05, 0.04, and 0.03 moles of ligand respectively (see Table 3.4a). It is not known whether the nucleotide-binding activity measured under these conditions represents the rate of exchange at a single site on the tubulin dimer or not. This question will be discussed in the final Chapter, in the light of experiments described below.

Similar experiments were also performed on guinea pig brain tubulin purified by the assembly of microtubules in vitro. The GTP-binding activity was found to be 11 - 15 times greater than the ATP-binding activity, instead of only twice, as in the experiments described above using tubulin purified by the batchwise method (see Table 3.4b). It is noteworthy that in tubulin preparations derived from microtubules, the ratio of GTP/ATP bound is remarkably constant despite large variations in the binding activities from preparation to preparation (Table 3.4b). Furthermore, it was found that aging selectively decreased colchicine- and GTP-binding, whereas the ATP-binding activity was stable: this might indicate the existence of separate sites for the binding of ATP and GTP

(see Table 3.4b). It should be pointed out that both GTP and glycerol were present throughout the purification procedure used to purify tubulin by microtubule assembly: under these conditions, tubulin was found to bind approximately 0.5 mole of colchicine, 0.25 mole of GTP and ca. 0.02 mole of ATP per mole of tubulin dimer (Table 3.4b).

These two sets of data strongly suggest that the GTP and colchicine binding activities, in particular, depend greatly upon the stability of the protein molecule, since dense compounds such as glycerol and sucrose, which are used to stabilise microtubules and tubulin (see 3.2.7), were also present in tubulin preparations derived from in vitro reassembled microtubules. The observed increase in the ratio of GTP/ATP bound to tubulin when the protein was purified by the polymerisation method suggests that the binding of GTP to tubulin is probably more specific than the binding of ATP. These data will be examined further in the following sections concerning the specificity of nucleotide-binding sites and the phosphorylation of tubulin.

### 3.2.6 Specificity of nucleotide-binding sites

Whether ATP is bound to the GTP-binding sites or to separate sites on tubulin is unclear. Further, it is possible that some of the minor protein impurities copurifying with tubulin could contribute to the total nucleotide binding capacity of the tubulin preparations employed. This necessitated further investigation of the specificity of the nucleotide-binding sites, which was initiated through competition experiments in which various derivatives of guanosine and adenosine nucleotides were

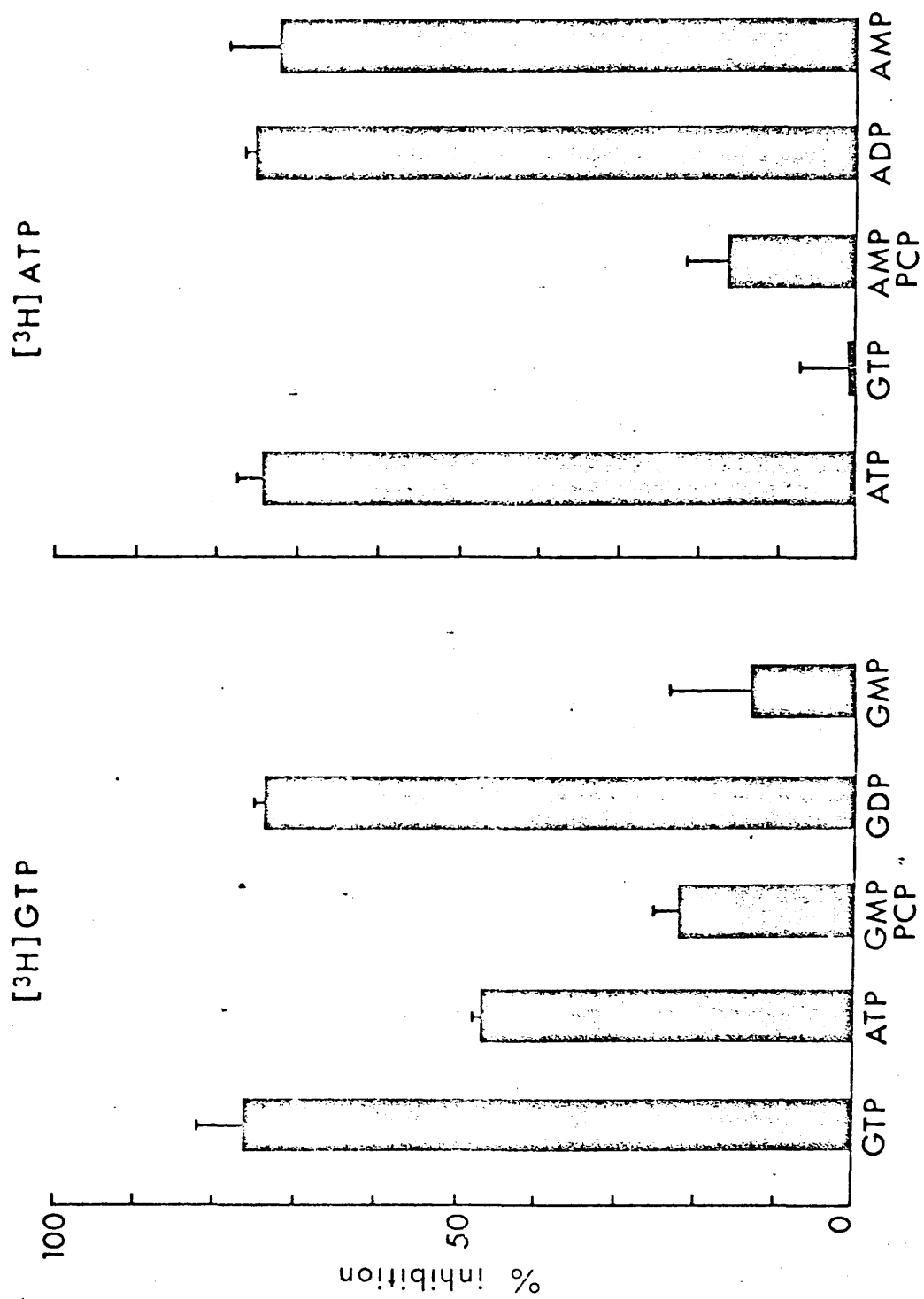
Table 3.5

The effects of unlabelled nucleotides on the binding of [ $^3$ H]GTP and [ $^3$ H]ATP to foetal rabbit brain tubulin

Labelled substrate	% of control binding activity						
[ $^3$ H]GTP (6.5 $\mu$ M)	plus	ATP	GTP	GMP-PCP	GDP	GMP	
	at 37°	53±1	23±6	78±3	26±1	77±10	
	at 4°	42±1	18±2	97±10	40±11	64±12	
[ $^3$ H]ATP (6.5 $\mu$ m)	plus	GTP	ATP	AMP-PCP	ADP	AMP	
	at 37°	99±6	26±3	84±5	25±1	28±6	

Reaction mixtures containing 100 - 200  $\mu$ g protein/tube were incubated 15 min at temperature indicated without or with added unlabelled nucleotide (100  $\mu$ M final concn.) and bound radioactivity was determined by Millipore filter assay as described in Methods section. Results are expressed as a percentage  $\pm$  S.D. (n = 3) of control values obtained in absence of added nucleotide.

Fig.3.7. Graphic presentation of data given in Table 3.5.





tested for their effects on the binding of  $\{^3\text{H}\}$  labelled GTP and ATP. The unlabelled compounds were added, at a final concentration of  $100\ \mu\text{M}$ , to incubation mixtures containing  $6.5\ \mu\text{M}$   $\{^3\text{H}\}$  labelled nucleotide, and the binding of the  $\{^3\text{H}\}$  - labelled species of either GTP or ATP was assayed by the Millipore filtration method (for method see Chapter II, 2.4.2). The effects of unlabelled nucleotides on binding activity are expressed as a percentage of the control (without added unlabelled nucleotide), in the same experiment. The results are summarised in Table 3.5 & Fig.3.7. In these experiments, it is assumed that the increased concentration (total) of nucleotides when 'cold' nucleotide derivatives are added do not substantially affect the nature of the saturation curves (see Table 3.5). It was found that at  $37^\circ\text{C}$ , both GTP and GDP competed with the binding of  $\{^3\text{H}\}$ GTP to tubulin; the activity was reduced to 23 % and 26 % of control (= 100 %) respectively (see Table 3.5). On the other hand, in the presence of GMP or of a non-hydrolysable analogue of GTP, GMPPCP ( $\beta,\gamma$  methylene-guanosine 5'- triphosphate; Miles Lab. Inc.), binding activity was only reduced to about 80 % of control. Under the same conditions, ATP decreased GTP-binding activity by 50 %. When incubations were carried out at  $4^\circ\text{C}$  in a parallel experiment, the effects of the added nucleotides differed to a small extent from those seen at  $37^\circ\text{C}$  (see in particular, effects of GDP and GMPPCP), although the overall pattern of results remained very similar (Table 3.5).

A study of the effects of 'cold' nucleotides on the ATP-binding activity of tubulin revealed that neither GTP nor AMPPCP ( $\beta,\gamma$ -methylene adenosine 5'-triphosphate; Miles Lab. Inc.) decreased the  $\{^3\text{H}\}$ ATP-binding activity to any significant extent.

Of the other adenosine nucleotides tested, ATP and ADP reduced [ $^3\text{H}$ ]-ATP binding by about the same extent (ca. 75 %), as was the case in the analogous tests for [ $^3\text{H}$ ]-GTP binding; 5'AMP also reduced binding at a similar degree, but in this case, the result is in marked contrast to that obtained when GMP was tested during [ $^3\text{H}$ ]-GTP binding.

From these experiments, the following tentative conclusions can be drawn concerning the specificity of the nucleotide-binding sites of tubulin.

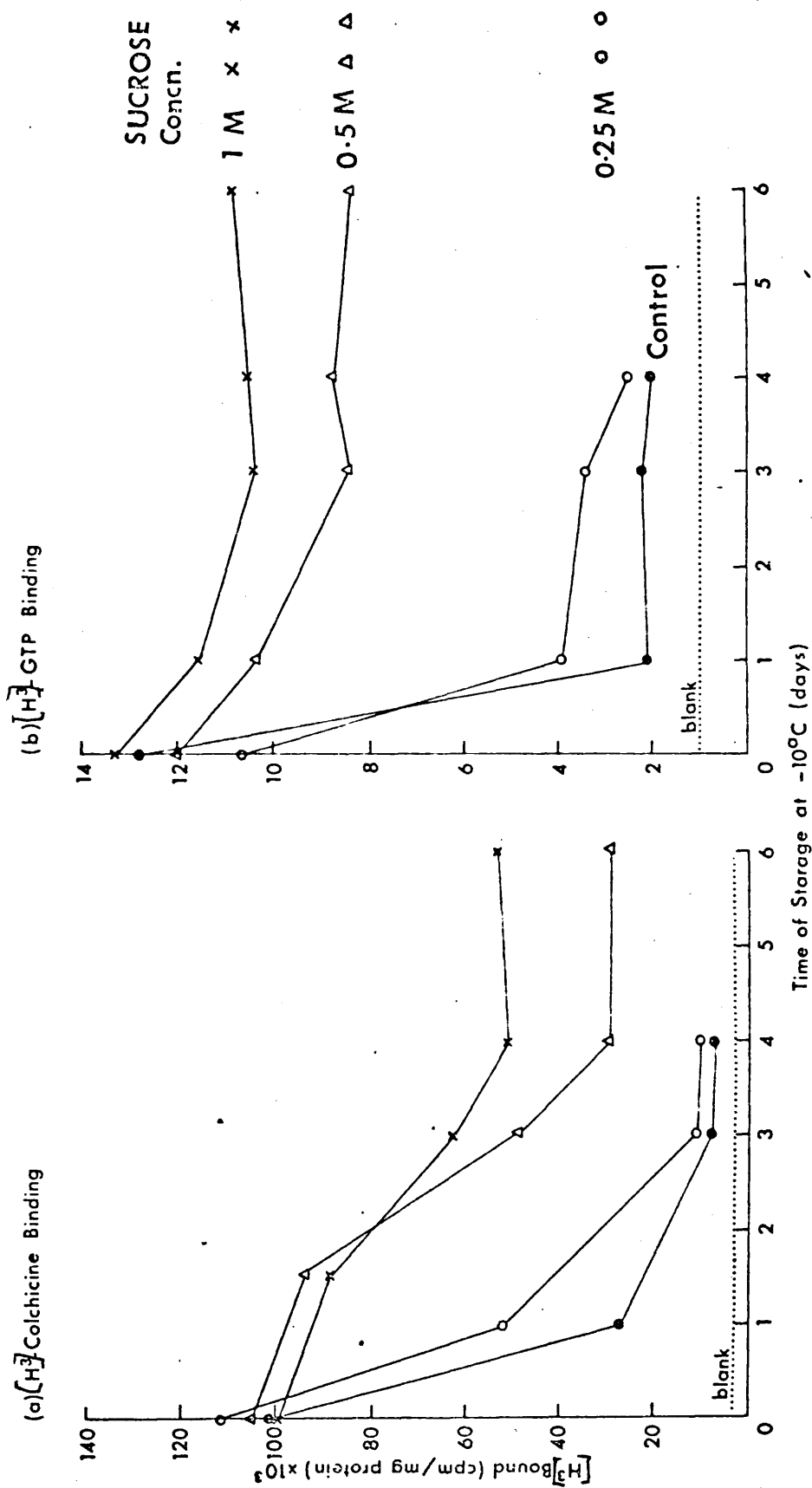
(1) Since ATP inhibits the binding of GTP, but GTP does not affect the binding of ATP to tubulin, the ATP-binding site(s) appear to be distinct from the GTP-binding sites.

(2) Since the non-hydrolysable analogues of GTP and ATP do not compete with the corresponding [ $^3\text{H}$ ] labelled nucleotide, it is likely that the hydrolysis of the  $\gamma$ -phosphate group can stabilise the binding of labelled nucleotides to tubulin.

(3) The lowering of the incubation temperature from 37°C to 4°C does not seem to affect the results of the competition for [ $^3\text{H}$ ]-GTP binding.

The nature of hydrolysis of the guanosine and adenosine triphosphates during the binding to tubulin might be of functional importance, and this may also be associated with the phosphorylation of tubulin by its intrinsic protein kinase activity (see section 3.4). Similar competition experiments using a doubly-labelled nucleotide triphosphates, e.g. [ $^3\text{H}$ ]-GTP[ $\gamma$ - $^{32}\text{P}$ ] would provide more information of this nature.

Fig.3.8. The effects of sucrose on the aging at  $-10^{\circ}$  of colchicine & GTP-binding activities of tubulin. (See text, 3.2.7. for details).



### 3.2.7 Effect of sucrose on colchicine- and GTP- binding activities

Sucrose was found to stabilise the colchicine-binding activity of tubulin (see Lee & Frigon, 1973). In parallel with the study of colchicine-binding activity, the decay of GTP-binding activity of tubulin in the presence of sucrose was investigated.

Foetal rabbit brain tubulin purified by the batchwise method was added to buffered incubation mixtures containing different concentrations of sucrose in 10 mM P-Mg buffer. The concentrations of sucrose in the buffered protein solution were 1 M, 0.5 M and 0.25 M and the samples were stored at  $-10^{\circ}\text{C}$  and assayed for both colchicine- and GTP- binding activity at various time intervals during storage for up to six days.

The same pattern of decay was observed for the two binding activities of tubulin (Fig.3.8). Sucrose, at a concentration of 1 M, exerted more effective protection against the decay of the binding activities than the other two lower concentrations tested. At a concentration of 0.25 M, sucrose provided very little protection, and after 3 days of storage, protein aggregates appeared, similar to those observed in the control sample without added sucrose. On the other hand, samples containing higher sucrose concentrations remained clear and were still active in binding colchicine and GTP after 6 days of storage. Sucrose, therefore, probably exerts a general stabilising effect on the whole protein molecule, rather than on specific binding sites.

### 3.3 Comparison on the binding properties of tubulin and of soluble 'transfer factors' isolated from pig brain

#### 3.3.1 Introduction

It has been shown that GTP reacts with the soluble transfer factors 'Tu' and 'Ts' (Elongation factors) required for protein synthesis in eukaryotic cells (Allende et al., 1967; Ertel et al., 1968). The reaction between GTP and the Tu factor is catalysed by the Ts factor, and the Tu-GTP complex so formed is found to be retained on Millipore filters (Allende & Weissbach, 1967). Shafritz & Weissbach (1969) reported that these factors could be isolated from calf brain by an ammonium sulphate fractionation procedure quite similar to that used in the batchwise method for purifying tubulin. It was therefore thought to be of interest to determine the extent to which a contamination of the tubulin preparations by soluble transfer factors might contribute to the GTP-binding activity of tubulin.

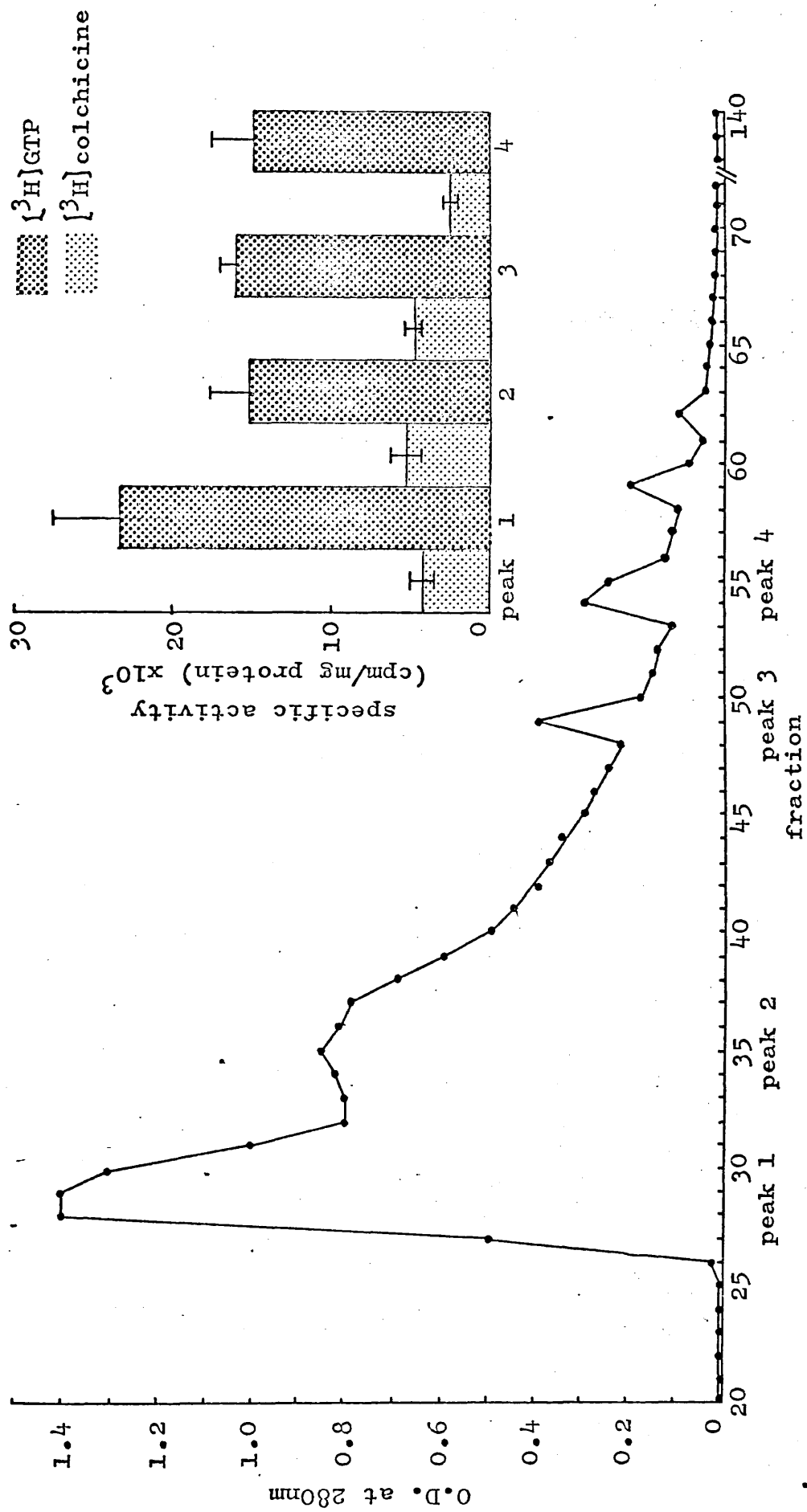
In these experiments, tubulin was purified from pig brain by the batchwise method of Weisenberg at al. (1968) in the presence of GTP (used to stabilise colchicine-binding activity) and soluble transfer factors were isolated at the same time from the same tissue by the method of Shafritz & Weissbach (1969). In order to facilitate comparison of the results obtained with both protein preparations, tubulin was also purified separately from pig brain in the absence of added GTP, or in the presence of dithiothreitol (without added GTP)

Legend to Fig.3.9.

Preparation of Tu & Ts factors

Pig brains ca.50 g were freed of blood vessels and meninges and homogenised with 2 vol of 10 mM-tris-HCl buffer, pH 7.4 containing 10 mM-NaCl, 10 mM-MgCl<sub>2</sub> and 1 mM-dithiothreitol (Tris-HCl buffer). All subsequent procedures were carried out at 4°C. The homogenate was centrifuged at 100,000 g for 2 h. To this high-speed supernatant solid ammonium sulphate was added to give 35 % saturation and the precipitate was removed by centrifugation for 20 min at 20,000 g. The resulting supernatant was made to 65 % saturation with solid ammonium sulphate, and the precipitate was pelleted by another centrifugation for 20 min at 20,000 g. The pellet containing the soluble transfer factors was redissolved in 6 ml of tris-HCl buffer and dialysed overnight against 2 litres of tris-HCl buffer. The dialysed material was fractionated by Sephadex G100 column chromatography. The column (2.5 x 75 cm) was eluted with tris-HCl buffer and 3 ml fractions were collected. The protein content of each fraction was determined spectrophotometrically at 280 nm. Four main protein-rich peaks were obtained (see Fig.3.9) and fractions constituting each peak were pooled. Samples were assayed for colchicine-binding activity by the DE 81 filter disc method and for GTP-binding activity, by the Millipore filtration method. In the assay for the GTP-binding activity of soluble transfer factors, the reaction mixture consisted 50 - 100 µg of protein, and 50 mM tris-HCl buffer, pH 7.4 containing 100 mM NH<sub>4</sub>Cl, 10 mM-MgCl<sub>2</sub>, 1 mM-dithiothreitol, and 6.5 µM-[<sup>3</sup>H]GTP (0.4 µCi). Incubations were performed at 0°C for 5 min as described by Shafritz & Weissbach (1969), and the reaction was stopped by dilution with 5 ml of cold tris-HCl buffer. The mixture was filtered on Millipore discs (HA 0.45 µ) and the discs were washed twice with 5 ml of cold tris-HCl buffer before determining the retained radioactivity as described in Chapter II, 2,4,2. Data for colchicine- and GTP- binding activity of each peak are shown in the inset of Fig.3.9.

Fig. 3.9.



(for method see Legend to Fig.3.9. and Chapter II, 2.2.1). The proteins present in the purified preparations were analysed by polyacrylamide gel electrophoresis; GTP and colchicine binding activities were studied in parallel for each preparation.

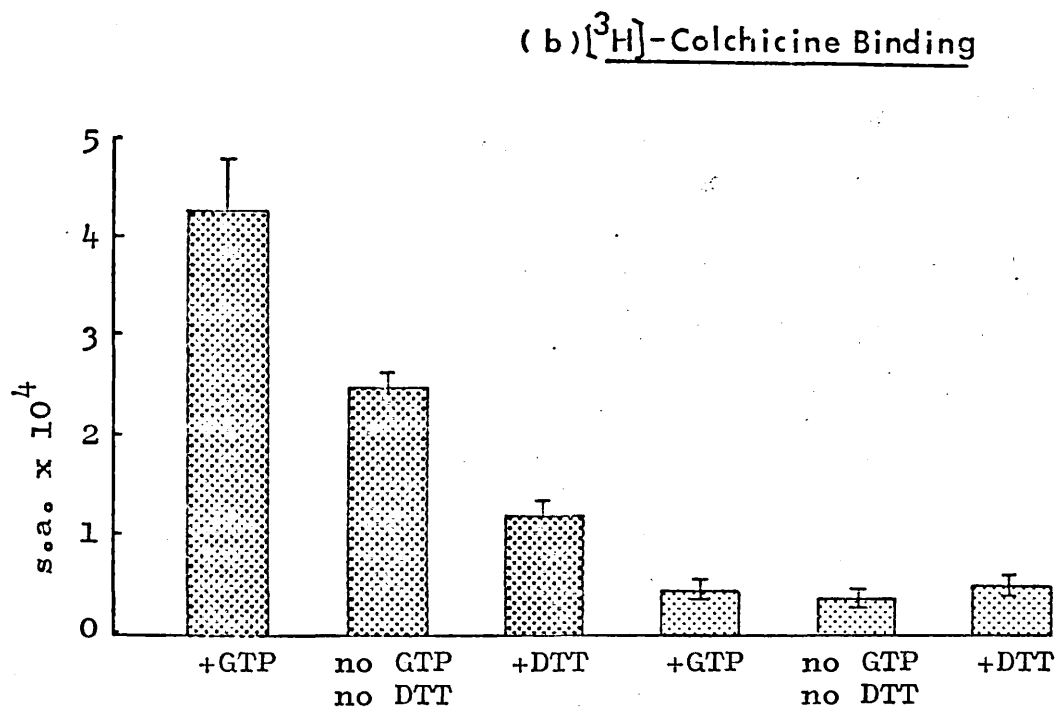
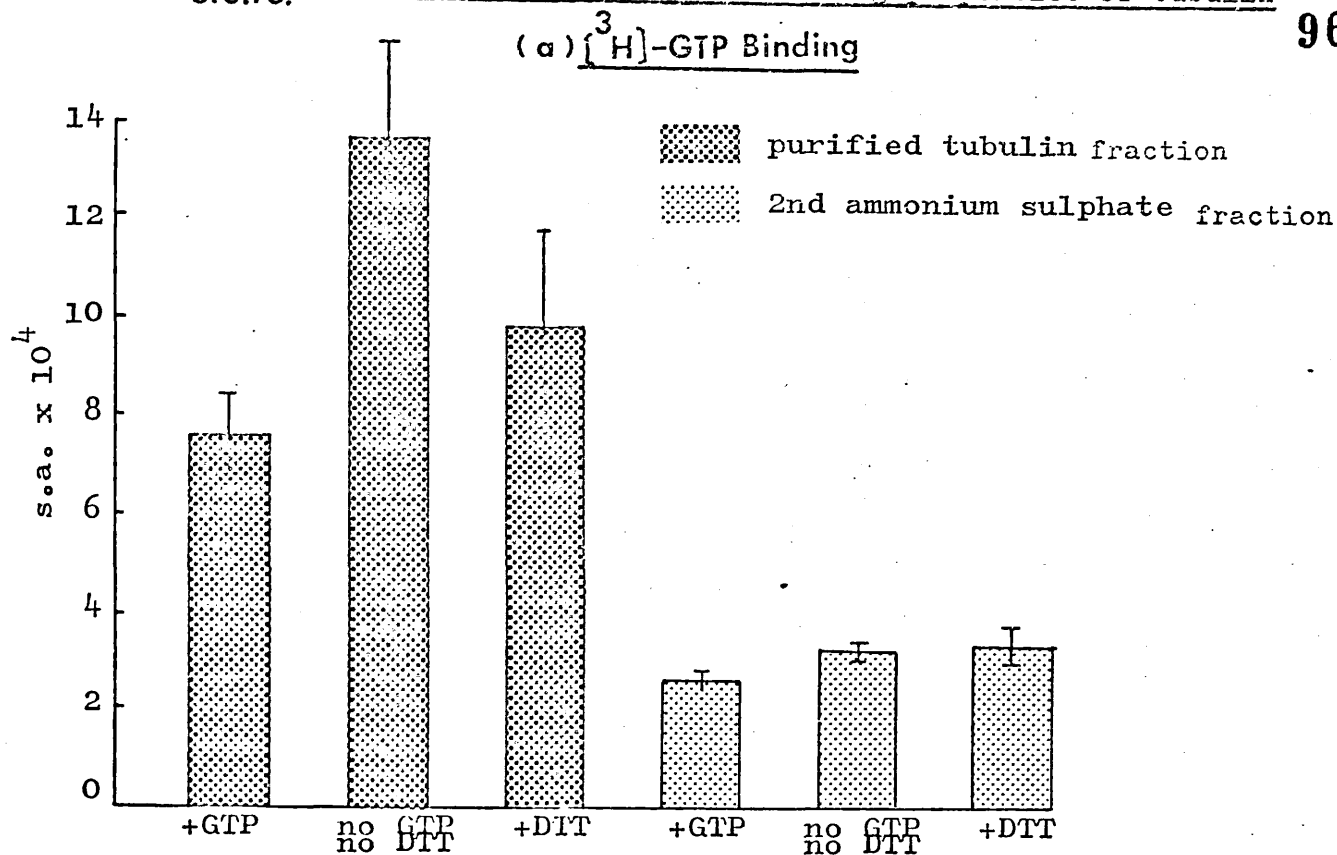
### 3.3.2 Results

The combined Tu and Ts factors were partially purified from pig brain extracts as outlined in the legend of Fig.3.9. It is worth noting at this juncture that during this preparation, no GTP is present in the buffers employed.

As can be seen in Fig.3.9, four main peaks of protein were eluted during chromatography on Sephadex G100 of the 2<sup>nd</sup> ammonium sulphate precipitate used as source of transfer factors all of which exhibited GTP-binding activity, (see inset, Fig 3.9). According to Shafritz & Weissbach (1969) transfer factors are concentrated in the first two peaks. It can be seen that the bulk of the protein was recovered in the first and second peaks, and that the specific activity for GTP-binding was found to be highest in the first peak, giving values about 35 % higher than those found for the material present in the three other peaks assayed, each of which bound GTP to about the same extent.

In fact, the data given in Fig.3.10b show that DTT and GTP, when included in the preparation buffers, had little effect on the colchicine-binding activity of crude tubulin preparations (i.e. in the second ammonium sulphate precipitate (3P)). However,





See text, section 3.3.2. for details.

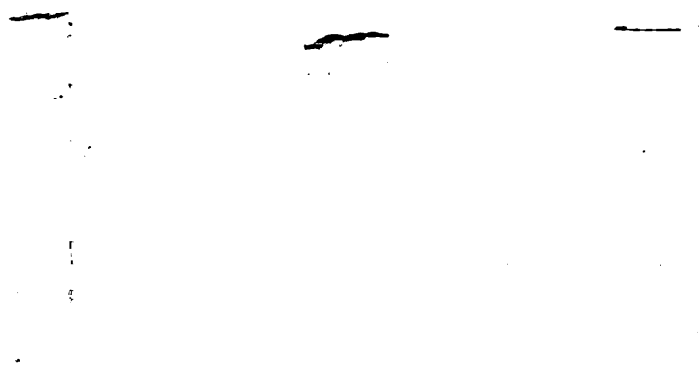
it is also clear from the data shown in Fig.3.10b that DTT caused inactivation when the purified tubulin prepared in its presence was assayed for colchicine-binding activity. In contrast, there was a marked increase in colchicine-binding activity, when tubulin was purified in the presence of added GTP (Fig.3.10b) confirming earlier reports showing that GTP stabilises the colchicine-binding site (Weisenberg et al., 1968). It is therefore apparent that while the colchicine-binding activity (cpm/mg of protein) in preparations enriched in Tu and Ts factors is about one-third of that in tubulin prepared under similar conditions (i.e., no GTP; plus DTT), and that tubulin prepared in the presence of GTP but without DTT, gives considerably higher values for colchicine-binding activity. (See inset, Fig.3.9 and Fig.3.10b)

With regard to GTP-binding, it is seen that DTT causes ~~small~~ small inhibition of GTP-binding to tubulin, as compared to the activity of samples purified in the absence of DTT or GTP (Fig.3.10a). The GTP-binding activity of tubulin prepared in the presence of DTT, but without added GTP, gave values of ca.  $10 \times 10^3$  cpm/mg, as compared with the values found for the first Sephadex peak of the Tu & Ts preparation (ca.  $25 \times 10^3$ ). Thus, both colchicine and GTP-binding activities of tubulin (prepared in the presence of DTT, but without GTP) give values of about one-third of those found for the transfer factor preparation in the first peak eluted during Sephadex chromatography. When tubulin was purified in the presence of GTP, however, the GTP-binding activity was similar to that of tubulin prepared in the presence of DTT. On the other hand, the GTP-binding activity of tubulin prepared in the absence of

Fig.3.11. Polyacrylamide gel electrophoresis patterns for peak fractions obtained during chromatographic purification of soluble transfer factors from pig brain

---

1<sup>st</sup> Peak                      2<sup>nd</sup> Peak                      Tubulin



See Fig. 3.9. and text section 3.3.2. for further details.

added GTP or DTT (column 2 of graph in Fig.3.10a) was definitely higher than that seen when GTP was included in the purification procedure. It was calculated that the specific activity of [<sup>3</sup>H]GTP present in the binding assay reaction mixture for the latter preparation should be approximately one-sixth of that of incubation mixtures containing tubulin isolated in the absence of added GTP. On this basis, one could therefore have expected considerably less binding than was in fact detected (see Fig.3.10a). These results suggest that GTP must have some stabilising effect on the GTP as well as on the colchicine-binding sites of tubulin.

The polyacrylamide gel electrophoresis patterns for the three types of tubulin preparation studied were identical, and when these are compared with the protein patterns for the main protein peaks obtained during chromatography in the preparation of soluble transfer factors (see Fig.3.11), it is apparent that the tubulin preparations contained little detectable protein comigrating with the main protein fractions seen during electrophoresis of soluble transfer factor preparations. Moreover, the soluble transfer factor preparation only contained a minor component comigrating with tubulin.

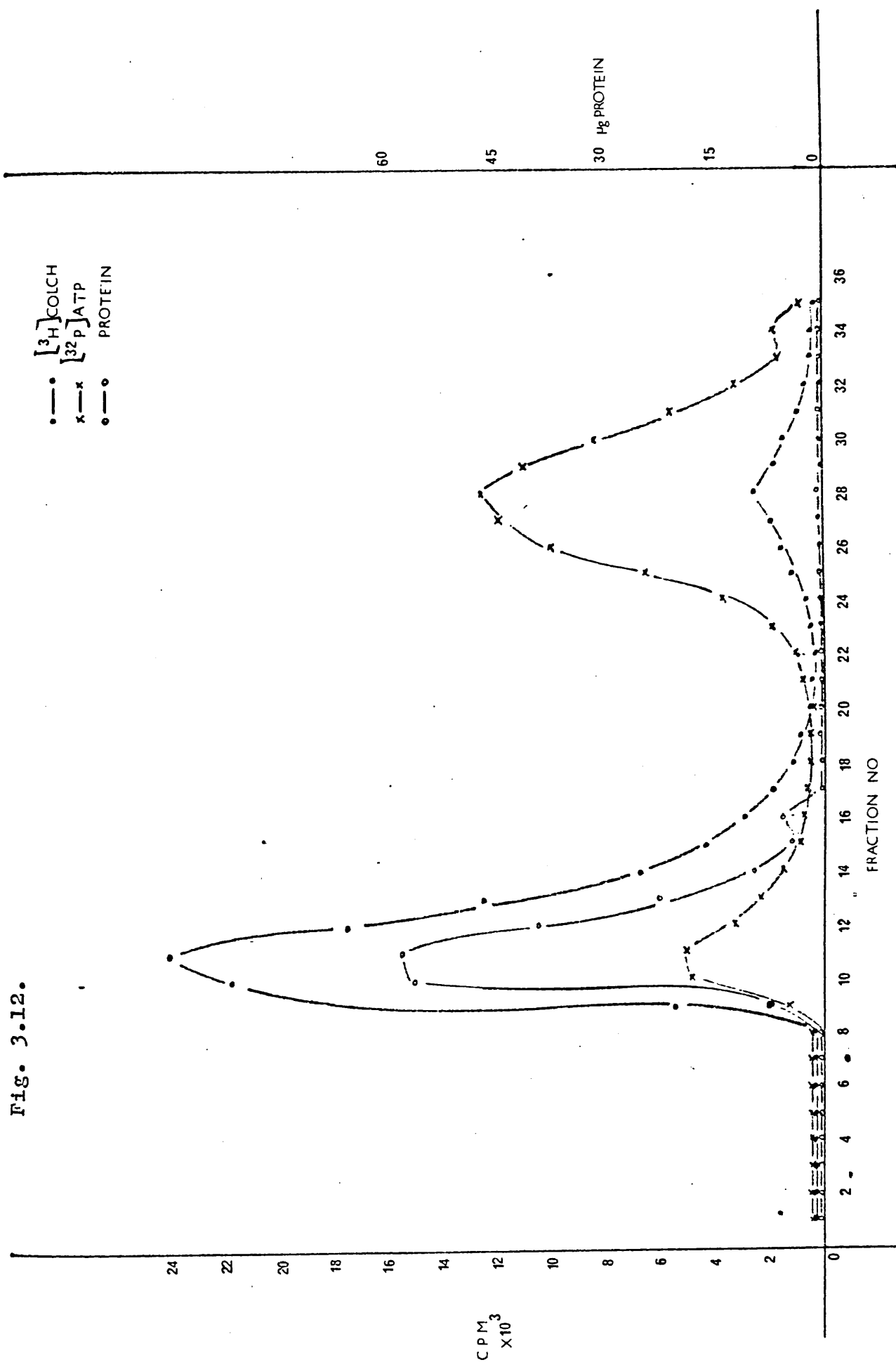
These data lead to the conclusion that crude tubulin preparation (second ammonium sulphate precipitate) may contain soluble transfer factors capable of binding GTP, but that purified tubulin does not contain such factors, and that the GTP-binding properties of the purified tubulin as described in previous sections do not appear to be related to any significant extent to the presence of soluble transfer factors as contaminants.

Legend to Fig. 3.I2

The in vitro binding of ( $^{32}\text{P}$ )ATP and ( $^3\text{H}$ ) colchicine to tubulin as determined by column chromatography on Sephadex G 100

---

Tubulin (1.5 mg of protein) which had been purified by the method of microtubule assembly (Shelanski et al. 1973) from guinea pig brain was incubated for 1h at  $37^\circ\text{C}$  with ( $^3\text{H}$ ) colchicine in P-Mg buffer, pH 6.8, followed by another 30 min of incubation with 10  $\mu\text{M}$  ATP( $\gamma\text{-}^{32}\text{P}$ ), (ca.  $10^6$  cpm) at the same temperature. The reaction was stopped by cooling on ice and samples were chromatographed on a Sephadex G 100 column (1x16 cm), which was eluted with P-Mg buffer. Fractions of 1 ml were collected and a 0.1 ml aliquot of each fraction was counted in 5 ml of Bray's scintillation mixture in both ( $^{32}\text{P}$ ) and ( $^3\text{H}$ ) channels.



### 3.4 Phosphorylation of tubulin in vitro and in situ

It has been shown by Goodman et al. (1970) that isolated calf brain tubulin possesses intrinsic protein kinase activity which is stimulated by cyclic AMP. These observations were confirmed and extended by Lagnado et al. (1972) for tubulin purified from pig, rabbit, guinea pig and rat brain (see also Soifer, 1972). The possibility that hydrolysis of the  $\gamma$ -phosphate group of the bound guanine nucleotide (found in isolated tubulin) (see 3.2.6) is related to the phosphorylation of tubulin remains to be examined.

In early experiments, guinea pig brain tubulin purified by the polymerisation procedure (2.2.3) was incubated with both [ $^3\text{H}$ ]-colchicine and [ $\gamma$ - $^{32}\text{P}$ ] ATP, and was subsequently chromatographed on Sephadex G100 column (1 x 16 cm). Protein-bound [ $^{32}\text{P}$ ] was associated with the peak containing the tubulin- $^3\text{H}$  colchicine complex (for details see Fig.3.12 and legend). Polyacrylamide gel electrophoresis of such preparations showed that at least part of the bound [ $^{32}\text{P}$ ] was covalently associated with the protein subunits (see Fig.3.13b.). In addition, the 2-3 bands near the origin of the gel, as well as protein components with molecular weights in the region of 36-45,000 migrating ahead of tubulin, were also radioactively labelled. These data further support the claims that tubulin is phosphorylated in vitro.

In situ phosphorylation of rat brain tubulin has been demonstrated after incubation of diced tissue (1 mm cubes) with radioactive [ $^{32}\text{P}$ ] orthophosphate for several hours (8-12 h)

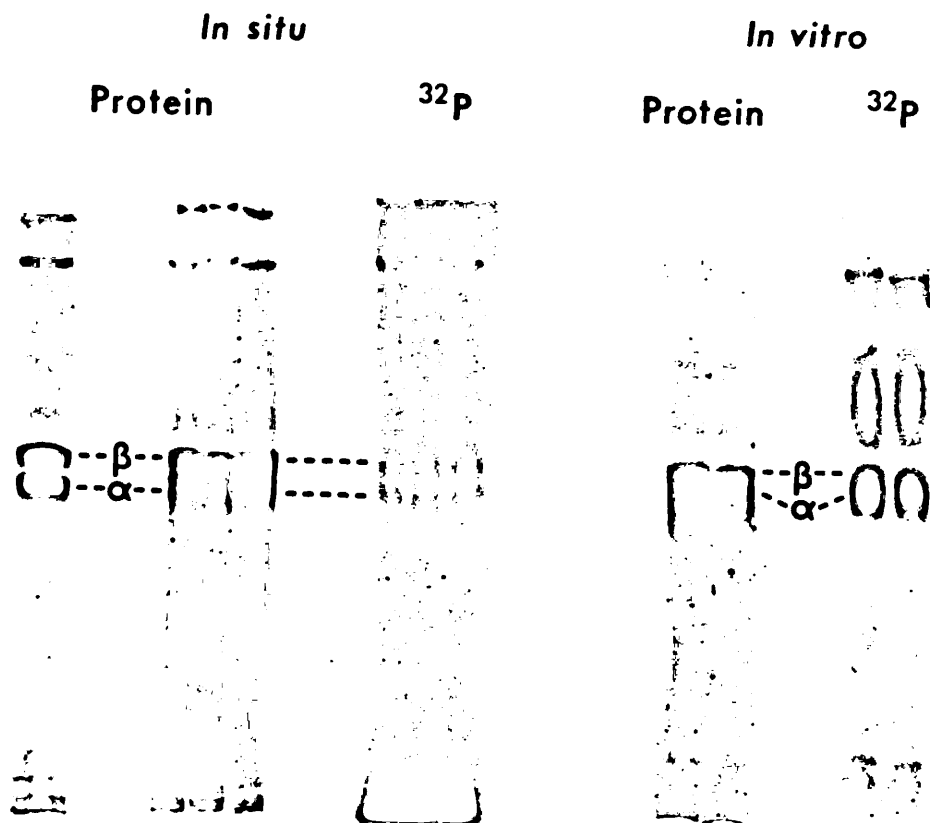
(Eipper, 1972), or after short (5-30 min) incubations of guinea pig cerebral cortex slices with [ $^{32}\text{P}$ ] orthophosphate; in the latter experiments tubulin was partly purified from extracts of incubated tissue either by the batchwise method or by precipitation with vincristine (Reddington & Lagnado, 1973). In both types of experiment, the specific labelling of tubulin was determined on material analysed by polyacrylamide gel electrophoresis.

In collaboration with M. Reddington, the phosphorylation of tubulin in situ has been re-examined using the in vitro reassembly of microtubules to purify tubulin from incubated tissue slices (Lagnado et al., 1975). Guinea pig cerebral cortex slices were incubated for 30 min at 37°C with [ $^{32}\text{P}$ ] orthophosphate (1 - 5 mCi) in oxygenated medium containing 128 mM-NaCl, 6.3 mM-KCl, 0.75 mM-CaCl<sub>2</sub>, 1.3 mM-MgCl<sub>2</sub> and 10 mM-glucose in 25 mM-tris-HCl buffer, pH 7.4. At the end of the incubation, the tissue was rinsed with ice-cold incubation medium and then homogenised in 10 mM-tris-HCl buffer, pH 7.4, to give a 20 % (w/v) homogenate. From the supernatant obtained after centrifugation of the homogenate at 100,000 g for 60 min microtubular protein was purified by the polymerisation procedure of Shelanski et al., (1973). The purified tubulin was then analysed by electrophoresis on SDS-polyacrylamide gels, using the tris-glycine buffered system (Chapter II, 2.5), and radioautography was carried out on slices of the stained and dried gels (2.5.6).

Under these conditions both the  $\alpha$  and  $\beta$  tubulin subunits were found to be labelled with [ $^{32}\text{P}$ ] (see Fig.3.13a), in agreement



Fig. 3.13. The phosphorylation in situ (a) and in vitro(b) of brain tubulin analysed by gel electrophoresis.



(a) Tubulin was isolated by the polymerisation procedure of Shelanski *et al.* (1973) from guinea pig cerebral cortex slices which had been incubated at 37°C for 30 min with [ $^{32}\text{P}$ ]orthophosphate (for details see text, 3.4.). Samples of tubulin were separated in polyacrylamide gel in tris-glycine buffer system (2.5.) and radioautography was carried out on dried slices of the stained gels. Protein stained with Coomassie Blue are shown for intact gel (left) and for triplicate slices of gel (middle), with corresponding radioautography (right;  $^{32}\text{P}$ ).  $\alpha$  &  $\beta$  denote tubulin subunits (see 3.4.). Note also heavy radioactivity associated with high molecular weight proteins near origin (bottom of picture).

(b) Rat brain tubulin (150  $\mu\text{g}$  of protein) purified by the polymerisation procedure of Shelanski *et al.* (1973) was incubated for 7 min at 37°C with 80  $\mu\text{M}$  ATP [ $^{32}\text{P}$ ] (10<sup>6</sup> cpm/0.2 ml reaction mixture) in 25-mM tris-HCl buffer, pH 7.4, containing 10 mM  $\text{MgCl}_2$ . Samples were analysed by polyacrylamide gel electrophoresis in a tris-glycine buffer system (see 2.5.2.). Duplicate slices of stained and dried gels are shown on the left-hand side (protein), and the corresponding radioautography are shown on the right-hand side ( $^{32}\text{P}$ ). For methods, see Chapter II, section 2.5.6. Origin is at the bottom of picture;  $\alpha$  &  $\beta$  indicate location of tubulin subunits. For details, see text, 3.4.

with results obtained when purified tubulin was labelled in vitro with [ $\gamma$ - $^{32}$ P] ATP (see Lagnado et al., 1972). Direct counting of 2 mm sections of the gel in 5 ml of Bray's scintillant showed that the  $\beta$ -subunit was preferentially labelled (about twice as much as the  $\alpha$ -subunit). The high molecular weight bands seen near the origin of the gel were also intensely labelled with [ $^{32}$ P]; in contrast with the phosphorylation in vitro, no radioactivity was associated with the faster-migrating minor protein bands (M.W. 36-45,000) (cf. Fig. 3.13a & 3.13b). Further, it was found that the radioactivity associated with tubulin constituted approximately 20 % of the total protein-bound [ $^{32}$ P] in the high-speed supernatant fraction (S1) (see also Reddington & Lagnado, 1973).

These data confirm and extend the previous findings that tubulin is a phosphoprotein which contains an intrinsic protein kinase activity. The functional role of phosphorylation of microtubular proteins (i.e. tubulin and dynein-like protein) remains to be clarified.

### 3.5 Phosphorylation of tubulin-like protein in synaptic membranes

Early work of Feit & Barondes (1970) and Lagnado et al. (1971) showed that a relatively high proportion of colchicine-binding activity present in rat brain homogenates was associated with a subcellular fraction enriched in synaptic membranes. This led to the suggestion that a more stable pool of membrane-bound tubulin exists in equilibrium with the labile pool of tubulin in the soluble fraction (Lagnado et al., 1971, 1974).

More recently, in collaboration with D. Jones & A. Matus,

Legend for Fig.3.14.

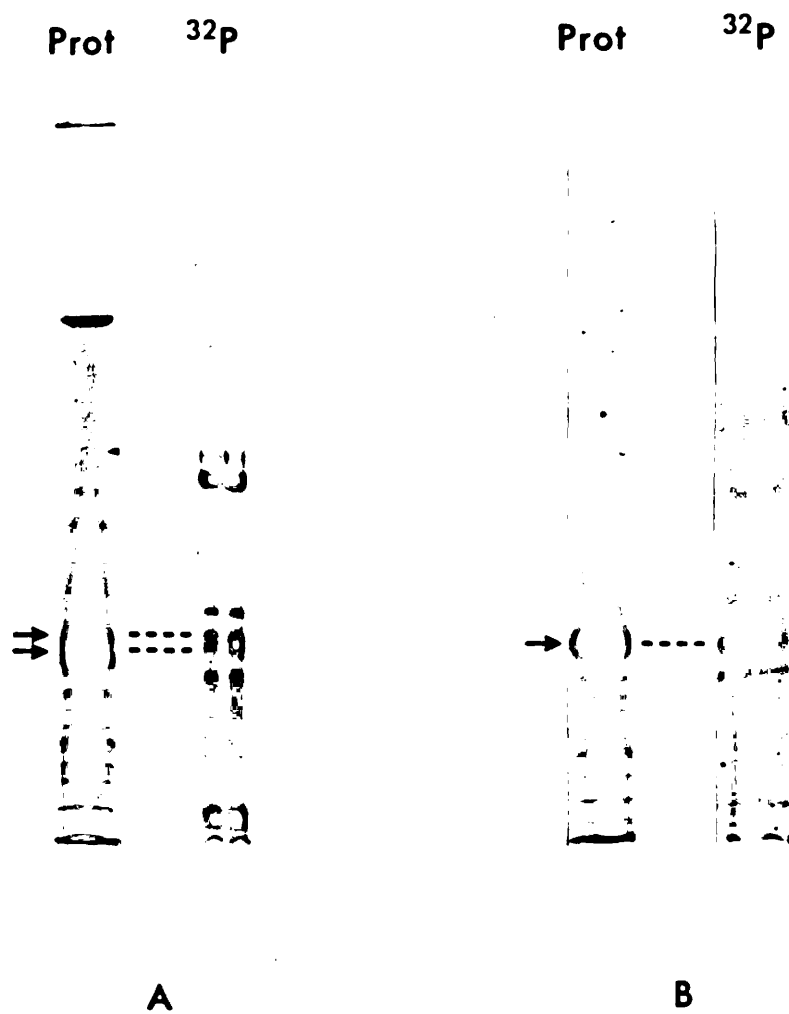
The phosphorylation in vitro of tubulin-like protein present in synaptic plasma membrane preparation from pig cerebellum.

Synaptic membranes were purified from pig cerebellum by the method of Jones and Matus (1974) (see also Chapter IV,4.2.5.1.) and phosphorylated by incubation in 25 mM-tris-HCl buffer (pH 7.4) containing 10 mM-MgCl<sub>2</sub> for 8 min at 37°C in the presence of 16 μM[γ<sup>32</sup>P]ATP (ca. 10<sup>6</sup> cpm/0.2 ml reaction mixture containing ca. 400 μg synaptic membrane protein). Reactions were terminated by heating samples for 5 min in boiling water bath in the presence of 0.5 % (final) SDS in 0.01-M tris-HCl buffer system (pH 6.8) containing 1.25-M urea (final) (Gels A), or in tris-HCl buffer system without urea (Gels B), and 50 μl samples were separated in SDS-polyacrylamide gels containing 5 M urea (A) or no urea (B), respectively. Electrophoresis was carried out in the discontinuous tris-glycine buffer system for sample A, or in a continuous tris-HCl system for sample B (see 2.5.2). Proteins stained with Coomassie Blue are shown for intact gels on left, and corresponding radioautographs of dried slices from duplicate stained gel are shown on right hand side (<sup>32</sup>P), arrows indicating location of tubulin-like protein band. Photograph of radioautograph in A reduced in size to compensate for apparent differences in mobilities due to different lengths of gel.

Fig.3.14.

In vitro phosphorylation of synaptic plasma membranes purified from pig cerebellum

---



it was found that highly purified synaptic plasma membranes isolated from pig brain cerebellum by the method of Jones & Matus (1974) are enriched in a protein component which comigrated as a single band with purified tubulin during polyacrylamide gel electrophoresis in a continuous tris-HCl buffered system (Fig.3.14B; for details see legend & Chapter II). In discontinuous tris-glycine buffered system containing urea (Chapter II, 2.5.2), tubulin split into  $\alpha$  and  $\beta$  subunits, and these were found to have comigrated with two protein bands present in synaptic plasma membrane preparations (Fig.3.14A) in which they constitute approximately 5 - 7 % of the total protein separated, as judged by densitometric analysis of the stained gel. This value agrees with earlier more indirect estimations based on colchicine-binding data for synaptic membrane preparations (Lagnado & Lyons, unpublished observation). In this work it was found that 30-40 pmoles colchicine were bound per mg of synaptic membrane protein.

It was of great interest to investigate the possibility that this tubulin-like protein in synaptic plasma membrane might be phosphorylated in vitro, as occurred in the case of soluble tubulin. Radioautography of the stained and dried gels of synaptic plasma membrane preparations which had been incubated with [ $\gamma$ <sup>32</sup>P]ATP (see Fig.3.14 & legend for details) showed that both the  $\alpha$  and  $\beta$  tubulin-like bands separated in the tris-glycine buffered system, and the single tubulin-like band seen in the tris-HCl buffered system, were radioactively labelled (Fig.3.14.A & B). When gels were sectioned and counted directly in 5 ml of Bray's scintillant, both the  $\alpha$  and  $\beta$

tubulin-like subunits were found to be equally labelled. These results are similar to those obtained when isolated tubulin was phosphorylated in vitro (see 3.4). The high molecular weight bands seen near the origin of the gels were labelled in both buffer systems; in addition, 2-3 minor bands migrating on either side of the tubulin-like protein were also labelled to a small extent (see Fig.3.14). Further, it was found that the addition of cyclic AMP to the incubation mixture did not seem to increase the phosphorylation of any of these protein components.

These results provide further evidence that tubulin is a component of the synaptic membrane; phosphorylation by intrinsic protein kinase activity may be associated with a functional role in the physiology of the synapse. Before such a role may be envisaged, however, more information concerning the dephosphorylation of tubulin and the structural relation of tubulin with other protein components in the synaptic membrane is needed.

### 3.6 Discussion

In this Chapter, it was shown that tubulin can be readily purified from foetal rabbit brain using methods similar or identical to those employed to isolate the protein from adult brain. Indeed, foetal brain appears to contain considerably more microtubular protein than adult brain on a wet weight basis, as judged by the recovery of colchicine-binding activity. These results confirm data reported by Bamberg et al. (1973) for developing chick brain. In the presence of urea and SDS, foetal rabbit brain tubulin splits into two fractions during polyacrylamide gel electrophoresis, of molecular weight 52,000 & 58,000 and these are referred to as the  $\beta$  and  $\alpha$  tubulin

subunits, respectively. These observations are in agreement with those reported for tubulin isolated from adult mammalian brain.

Vincristine was found to stabilise the colchicine-binding activity of foetal brain tubulin, and this suggested possible interactions between each type of binding site. Experiments to investigate this possibility will be described in Chapter IV & V. In addition to the GTP-binding sites, an independent ATP-binding site(s) has been detected, and indirect evidence shows that the binding of the guanosine and adenosine triphosphates to tubulin is probably accompanied by the hydrolysis of the  $\gamma$ -P group. It has been reported that both GTP and ATP can act as phosphate donors during the phosphorylation of tubulin by its associated protein kinase (Soifer, et al., 1972; Lagnado, Tan & Reddington, 1975; Piras & Piras, 1974). It is possible that the hydrolysis of either of these two nucleotide triphosphates is related to the transphosphorylation reaction which is thought to occur between the two bound guanosine nucleotides (Berry & Shelanski, 1972; Jacobs et al. 1974).

Experiments on the phosphorylation of tubulin carried out on both in vitro and in situ systems confirm and extend early findings that both tubulin subunits are phosphorylated and that the  $\beta$  subunit is preferentially phosphorylated in situ. However, Eipper (1972) reported that only the  $\beta$ -subunit was phosphorylated. The reasons for this discrepancy is unclear, but might be related to the different experimental conditions used to label the protein (short incubation vs tissue culture for 11 h) or to isolate tubulin (polymerisation

vs chromatographic fractionation) employed in the two studies.

The identification of a tubulin-like protein in synaptic plasma membrane preparations purified from pig cerebellum lends support to indirect evidence for the presence of membrane-bound tubulin (Feit & Barondes, 1971; Lagnado et al., 1972). These preparations also bind colchicine and vincristine, and can be phosphorylated in vitro. A very recent report by Piras and Piras (1974) shows that tubulin from chick embryonic muscles and from Hela cells can be phosphorylated in intact cells; tubulin present in adult tissue was a poor substrate for phosphorylation in situ. It was suggested that phosphorylation of tubulin may be a general phenomenon (Piras & Piras, 1974). However, the presence of tubulin as a main component of synaptic membranes, where it is readily phosphorylated in vitro may indicate a more specific functional role for tubulin phosphorylation in connection with some aspects of synaptic function.



Chapter IV      The binding of [ $^3\text{H}$ ]-vincristine to tubulin

4.1      Introduction

4.2      Methods

4.2.1      Starting material for the purification of [ $^3\text{H}$ ]-vincristine

4.2.2      Purification of [ $^3\text{H}$ ]-vincristine from the crude labelled product

4.2.3      Determination of purity of [ $^3\text{H}$ ]-vincristine

4.2.3.1    Chromatographic analysis

4.2.3.2    Spectrophotometric analysis

4.2.3.3    Benzene extraction of [ $^3\text{H}$ ]-vincristine

4.2.3.4    Recovery of [ $^3\text{H}$ ]-vincristine

4.2.4      [ $^3\text{H}$ ]-vincristine-binding assay

4.2.5      Subcellular fractionation

4.2.5.1    Methods of fractionation

4.2.5.2    Assay of marker-enzymes:

(a)    Lactic dehydrogenase

(b)    Fumarase

(c)    Acetylcholine esterase

4.3      Results

4.3.1      The binding of vincristine to microtubular protein

4.3.2      Vincristine-induced precipitation of microtubular protein

4.3.3      Characteristics of [ $^3\text{H}$ ]-vincristine-binding to purified tubulin

4.3.4      The effects of colchicine and GTP on the binding of [ $^3\text{H}$ ]-vincristine to tubulin

4.3.5      Subcellular distribution of [ $^3\text{H}$ ]-vincristine and [ $^3\text{H}$ ]-colchicine-binding receptors

4.4      Discussion

## Chapter IV      The binding of [<sup>3</sup>H]-vincristine to tubulin

### 4.1      Introduction

The properties of colchicine- and GTP-binding sites of tubulin from brain were dealt with in previous Chapters. In the present Chapter, studies are reported concerning the binding of vincristine to tubulin with a view to obtaining a more integrated view of the properties of and possible relationships among the three types of binding sites. These studies were made possible by recent success in the labelling of vinblastine (Beer et al., 1964; Greenius et al., 1968) and vincristine (Owells & Donigian, 1972) with tritium. Using these materials, Owells et al., (1972) and Wilson et al., (1974) have published preliminary data concerning the binding of Vinca alkaloids to chromatographically purified brain tubulin. This work has now been extended using microtubular protein purified by the polymerisation procedure and [<sup>3</sup>H]-labelled vincristine. In addition, the distribution of vincristine-binding sites in subcellular fractions from rat brain was investigated and compared with the distribution of colchicine-binding receptors.

### 4.2      Methods

Since pure [<sup>3</sup>H]-vincristine is not obtained commercially, unlabelled vincristine was tritiated by the Radiochemical Centre, Amersham, and the crude mixture so obtained was subsequently purified and analysed as described below.

#### 4.2.1 Starting material for the purification of [<sup>3</sup>H]-vincristine

The method used for tritium labelling of vincristine was that of Owellen & Donigian (1972). Unlabelled vincristine sulphate (50 mg starting material; supplied by Eli Lilly Co. Ltd., Basingstoke) was reacted with 25 Ci of [<sup>3</sup>H]-trifluoroacetic acid. After removal of the labile tritium, the crude product (50 mCi) was dissolved in 40 ml of 0.025 N sulphuric acid and stored at 2° C to minimise decomposition by self-radiolysis.

#### 4.2.2 Purification of [<sup>3</sup>H]-vincristine from the crude labelled product

Tritiated vincristine was purified from the crude product by chromatography on a cellulose phosphate column essentially as described by Beer et al. (1964).

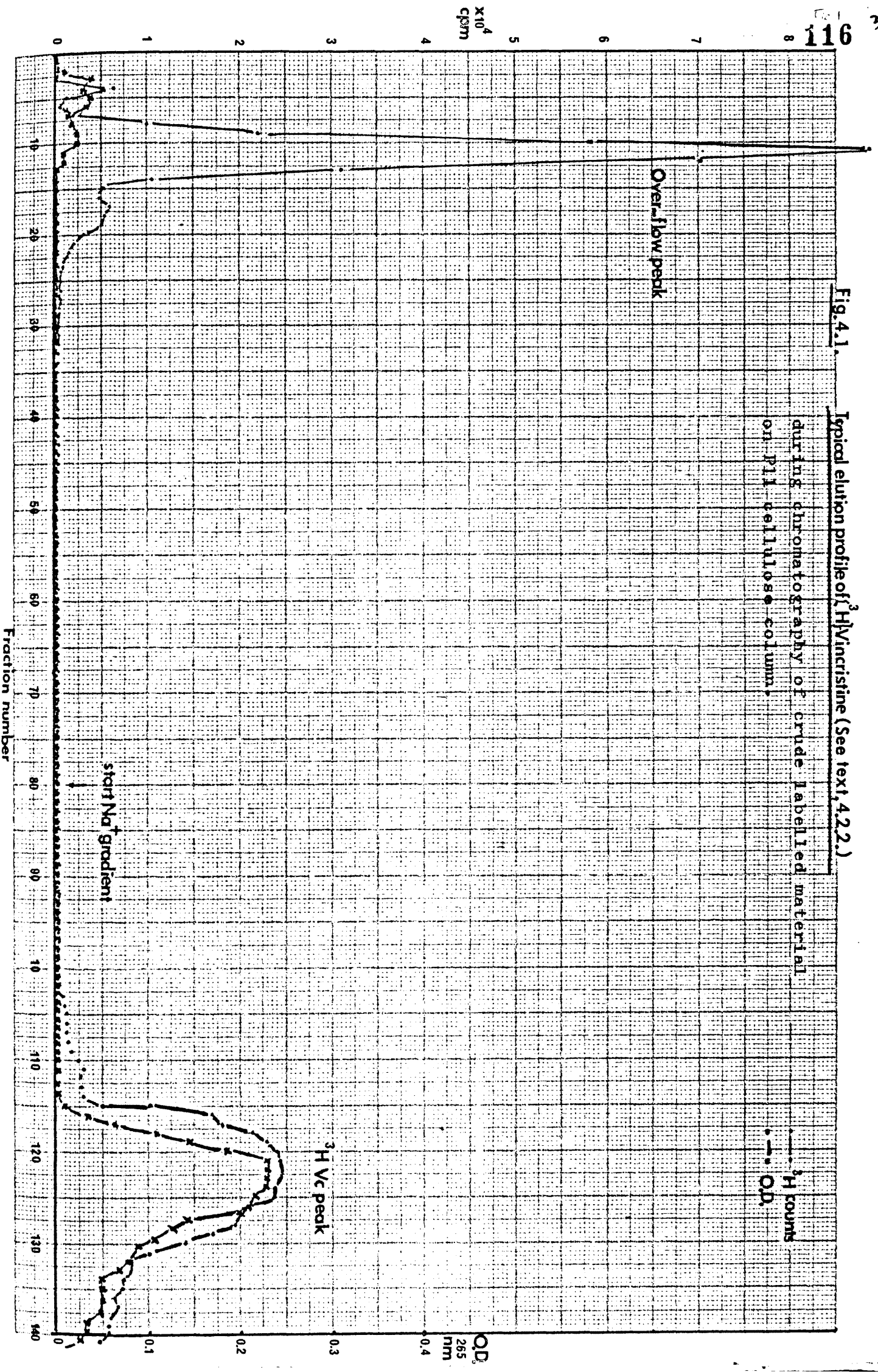
About 30 g of cellulose phosphate (Whatman P11) were suspended in 500 ml of 0.5 N HCl overnight. A chromatographic column (1.5 x 25 cm; Wright Scientific Ltd.) was packed with the acid-treated P11 cellulose phosphate using a constant flow of 0.5 N HCl. After packing, this solution was replaced by 0.05 M-NaH<sub>2</sub>PO<sub>4</sub> buffer which had been adjusted to pH 3.4 with orthophosphoric acid. The column was mounted on a refrigerated LKB fraction collector and the whole system was maintained at 4° C. The column was allowed to equilibrate completely with 0.05 M-NaH<sub>2</sub>PO<sub>4</sub> buffer. This normally required 1.5 litre of buffer and took 2 days at a flow rate of about 12 drops per min. After this period, the column measured approximately 22 cm. The crude labelled vincristine (2.5 mCi in 2 ml) was made dense by adding 0.1 g of lactose and loaded onto the column. The column was then washed with 0.5 litre of the

$\text{NaH}_2\text{PO}_4$  buffer to remove completely unbound radioactive material. The effluent was continuously monitored at 280 nm by means of a UV recorder (LKB Uvicord) and 0.1 ml samples were taken from the fractions (4 ml each) collected for tritium counting in 5 ml of Bray's scintillant. Washing was continued until the radioactivity of the eluates reached a constant level. The bound radioactive vincristine was then eluted with 0.05 M-  $\text{NaH}_2\text{PO}_4$ , pH 3.4 containing 0.05 M NaCl. Approximately 0.5 litre of buffer was required to displace the bound radioactive material which was eluted as a single peak (Fig.4.1), confirming the results described by Owellen et al. (1972b). The fractions constituting this peak were pooled and freeze-dried, and the residue was dissolved in 3ml of double glass distilled water and stored in a dark bottle at 4° C.

In the process of chromatography on P11 cellulose phosphate, the column was usually overloaded with crude labelled product. Excess radioactive components emerged as the "over-flow" peak during the initial elution with 0.05 M- $\text{NaH}_2\text{PO}_4$  buffer, pH 3.4. No attempt was made to reprocess this excess material due to its large volume, but the amount loaded was decreased by half in subsequent purifications. In the experiment described (Fig.4.1), about 200  $\mu\text{Ci}$  of [ $^3\text{H}$ ]-vincristine was recovered: this represents approximately 10 % of the total added radioactivity. This low recovery was probably due to excessive loading of the P11 cellulose phosphate column as suggested by the high "over-flow" peak (see Fig.4.1). This was also reflected by a further increase in recovery of about 20 % during subsequent purification, when half the amount (1.25 mCi in 1 ml) of crude labelled product was used, bringing

Fig. 4.1. Typical elution profile of [<sup>3</sup>H]vincristine (See text, 4.2.2.)

during chromatography of crude labelled material on P11 cellulose column.



— H counts  
- - - OD

<sup>3</sup>H Vc peak

Overflow peak

Start Na<sup>+</sup> gradient

Fraction number

OD,  
265  
mm

x10<sup>4</sup>  
cpm

the total recovery of radioactive material in the vincristine peak to approximately 30 %. Since only about 30 - 35 % was eluted in the "overflow" peak, approximately 30 - 40 % of the added radioactive material remained bound to the column after elution with 0.05 M NaCl. No attempt was made to characterise these bound radioactive impurities.

#### 4.2.3. Determination of purity of $\{^3\text{H}\}$ - vincristine

Before use, the purity of  $\{^3\text{H}\}$  -vincristine isolated as described above was tested by the analytical procedures described below.

##### 4.2.3.1. Chromatographic analysis

Chromatography was carried out on silica gel coated glass plates (gel 3 mm thick) or on Eastman Kodak chromatogram sheets (6061), and in three different solvent systems. Radioactive alkaloids (5  $\mu\text{l}$  containing approximately  $120 \times 10^3$  cpm) and unlabelled vincristine (20  $\mu\text{g}$ ) were applied to the plates which were separately developed in three solvent systems. The plates or chromatogram sheets were dried and sprayed with Dragendroff's reagent prepared according to the method of Munier & Macheboeuf (1951). Dragendroff's reagent was prepared by mixing equal volumes of the two following solutions:

Solution A: 0.85 g bismuth nitrate, was dissolved in 10 ml of glacial acetic acid and diluted to 50 ml with water.

Solution B: 8 g of potassium iodide dissolved in 20 ml water.

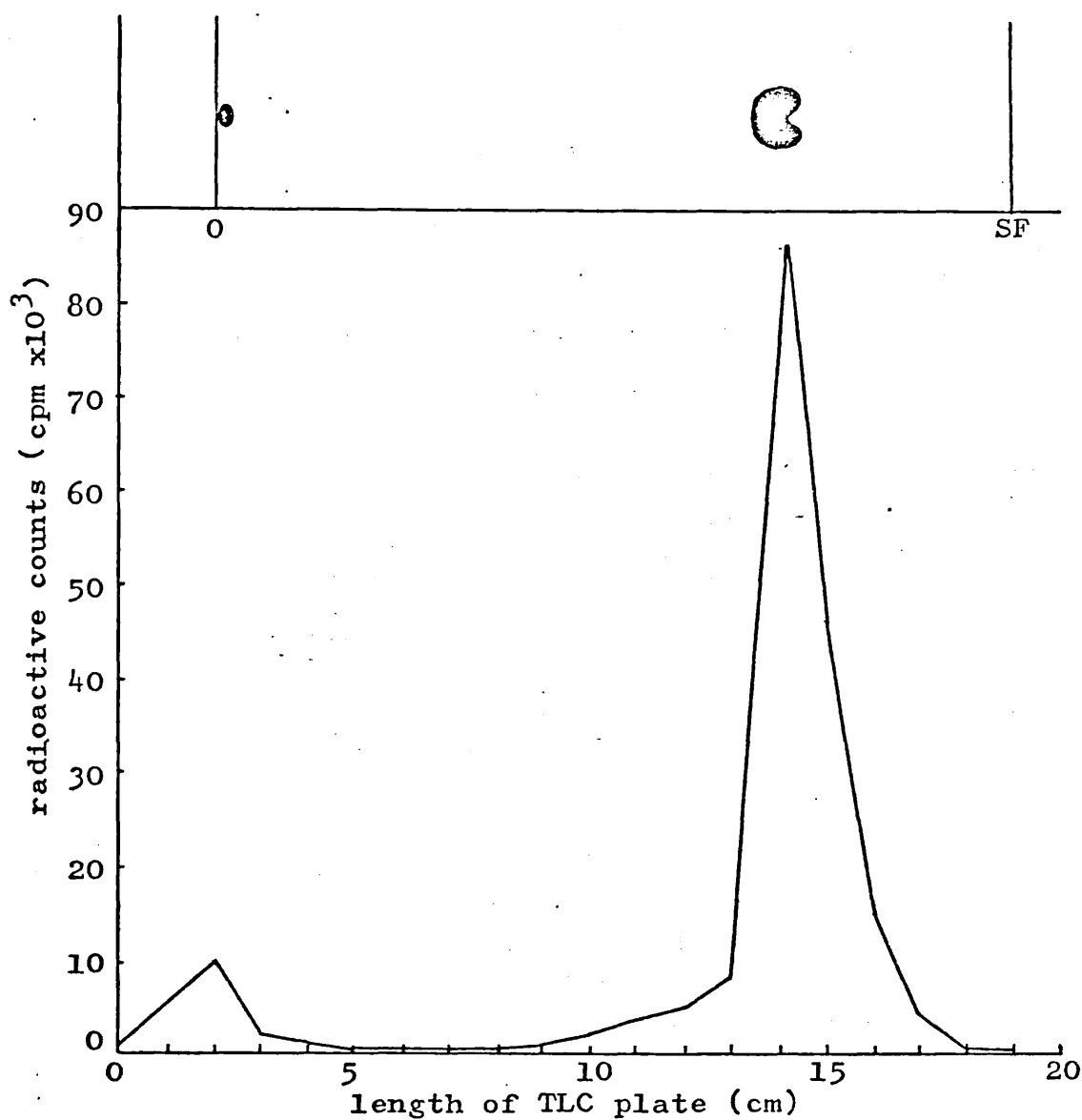
For Spraying, 2 ml of glacial acetic acid were added to 1 ml

of Dragendorff's reagent, and the solution was diluted with 10 ml water. It was relatively stable when stored in the dark. All Vinca alkaloids tested reacted with this reagent to give an orange colour. It was found that the optimum sensitivity range of this reagent for Vinca alkaloids, as detected after thin-layer chromatography, was between 10 and 50  $\mu\text{g}$ . Below this range only very faint colour could be seen and below 0.2 g of applied sample, no colour could be detected. When 80  $\mu\text{g}$  of Vinca alkaloid was applied, a large and widely spread spot was obtained. The Rf values of various alkaloids in different solvent systems are given in Fig.4.2. The chemical structures of these compounds is shown in Fig.6.12, Chapter VI.

The strip of silica gel containing the alkaloid spot was divided vertically into 1 cm sections, and each section of silica gel was scraped into 5 ml of Bray's scintillant for counting. When Eastman Kodak chromatogram sheets were used, the strips were 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 materials, which decreased the counts by 10 - 20%. Radioautography was carried out by exposing the chromatogram to X-ray film for at least three weeks.

In all three solvent systems used, 90 - 95 % of the radioactivity in the [ $^3\text{H}$ ]vincristine purified by the column chromatography (see above) was found to co-migrate with authentic vincristine. Approximately 5 - 10 % of the radioactivity remained at the origin. A typical chromatographic separation of [ $^3\text{H}$ ]-vincristine is shown in Fig. 4.2 together with the radioactivity distribution profile. Radioautographic analysis confirmed the above findings.

(see text,4.2.3.1)



solvent system:- chloroform:methanol 1:1

Thin layer chromatography of Vinca alkaloidsRunning solventRf values

	<u>Vinblastine</u>	<u>Vincristine</u>	<u>Leurosine</u>	<u>Leurosidine</u>
Chloroform :Methanol 1:1	0.892	0.782	0.906	0.872
Ethylacetate : Ethanol 3:1	0.332	0.338	0.578	0.210
Acetone	0.495	0.522	0.591	0.125
N-propanol : water 7:1	0.792	0.634	0.805	0.610



#### 4.2.3.2 Spectrophotometric analysis

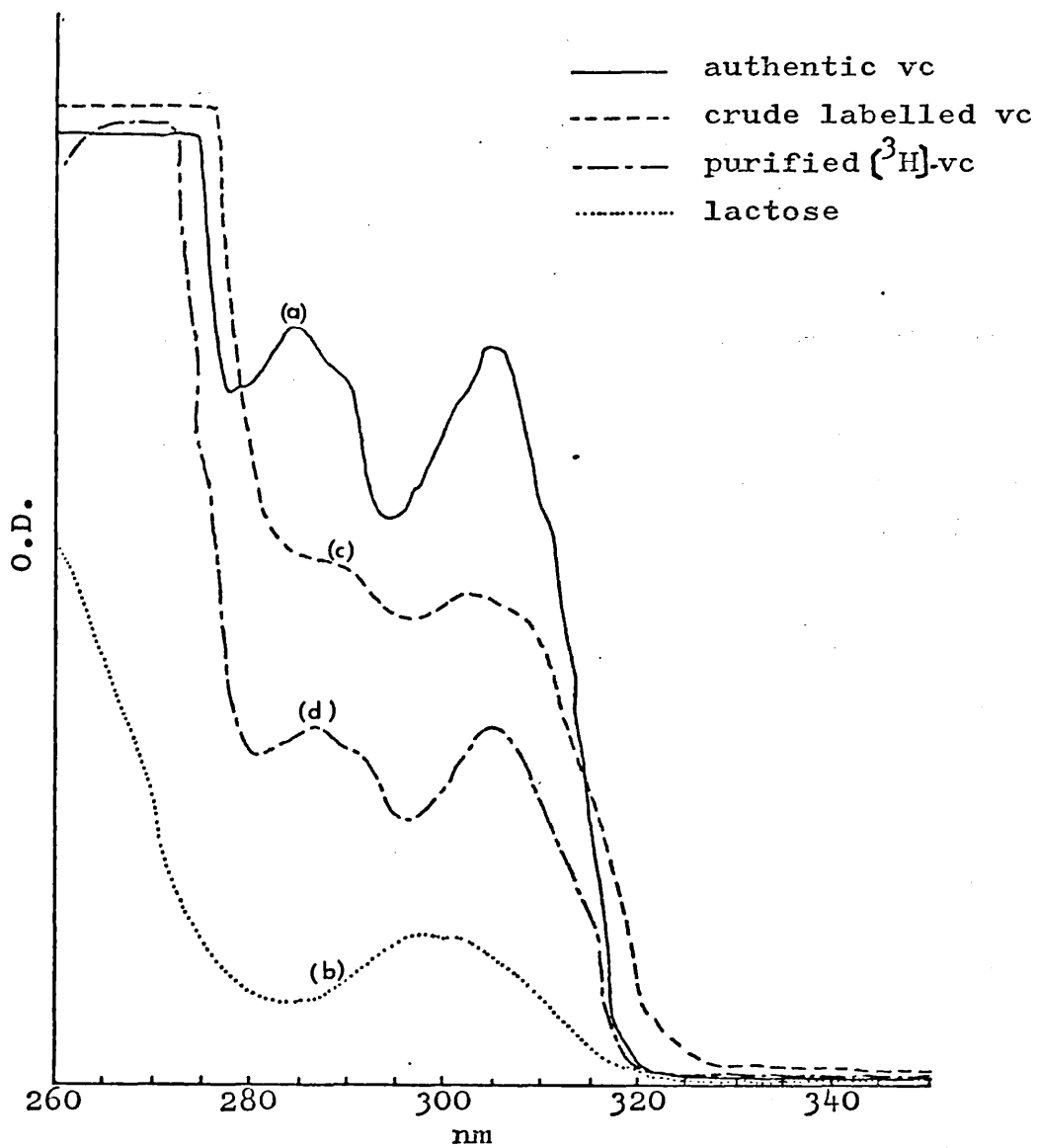
Scanning of authentic vincristine in the UV region showed two distinct absorption peaks, at 305 nm and 285 nm, and a high absorption peak at 265 nm (see Fig. 4.3a). L-lactose, which is present as a stabilising agent (10 parts /part of alkaloid) in the vincristine preparations used, does not interfere with these absorption peaks, as shown in Fig.4.3b. A comparison of the UV scans obtained for both the crude labelled product and the purified [ $^3\text{H}$ ]-vincristine with that for authentic unlabelled vincristine indicates that [ $^3\text{H}$ ]-vincristine was relatively more enriched in vincristine than the crude labelled product (see Fig. 4.3d & e).

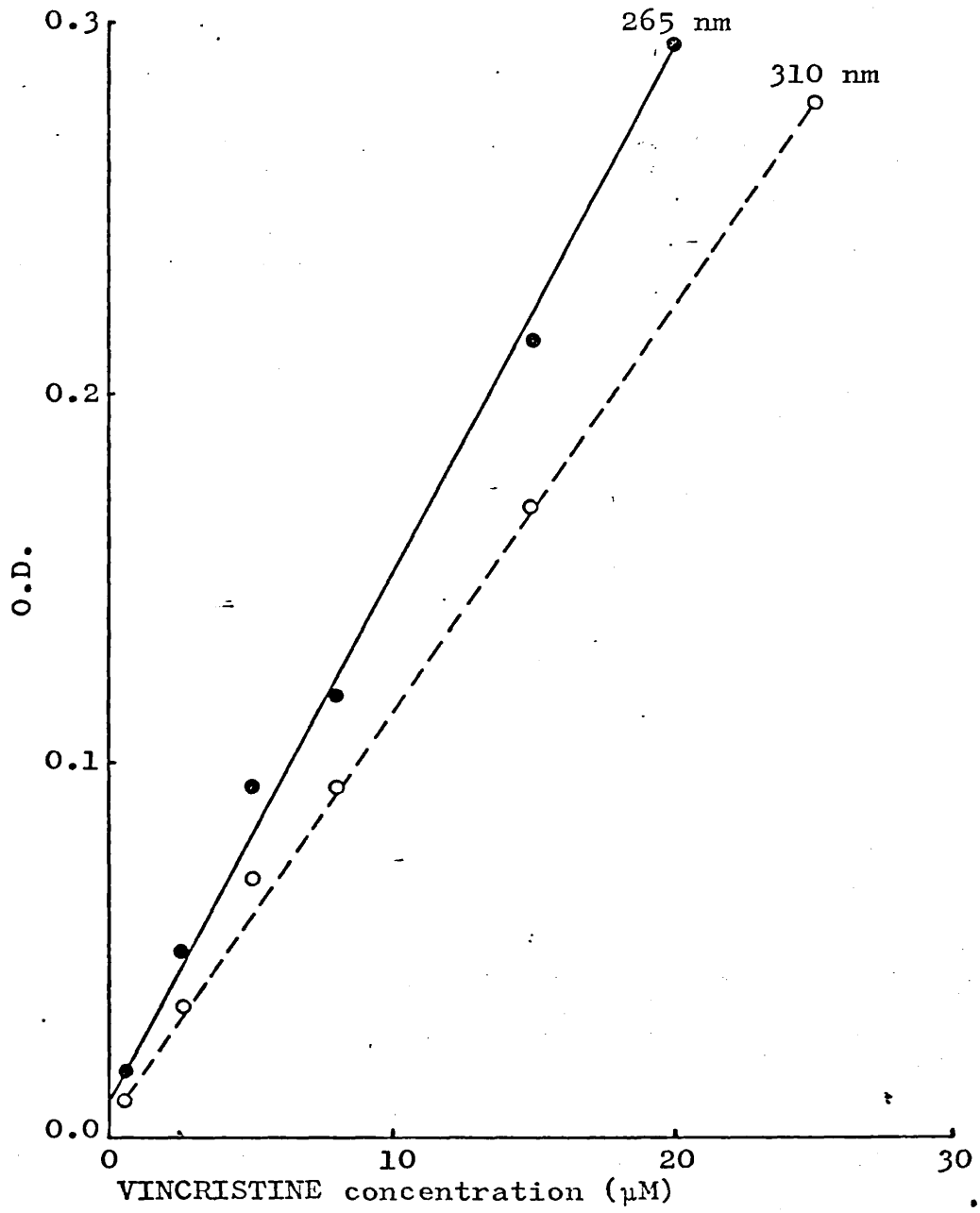
The concentration of the purified [ $^3\text{H}$ ]-vincristine was determined from calibration curves (Fig. 4.4) in which absorption of authentic vincristine was plotted as a function of concentration at 310 nm and 265 nm. In three different preparations, it was found that the concentration of the purified [ $^3\text{H}$ ]-vincristine gave values between 20 and 50  $\mu\text{M}$ .

#### 4.2.3.3 Benzene extraction of [ $^3\text{H}$ ]-vincristine

Vincristine is easily extracted in benzene in contrast to tritiated water and other compounds such as [ $^3\text{H}$ ]-trifluoroacetic acid present in the crude product. Samples (5  $\mu\text{l}$ ) of known radioactivity were each diluted with 0.5 ml water and extracted with 3 ml of benzene. The benzene-water mixtures were shaken for 30 min, and the two layers were allowed to separate by standing for 15 min. Aliquots of the benzene layer (top layer) were counted in 5 ml of Bray's scintillant and the total radioactivity

vincristine (see text, 4.2.3.2.)





extracted could be calculated. After extraction of the purified [ $^3\text{H}$ ]-vincristine preparation with benzene  $92 \pm 4\%$  (average for three separate preparations) of the counts were recovered in the organic phase, as compared to  $65 \pm 4\%$  found for the crude labelled material. This further supports the purity of the final product.

#### 4.2.3.4 Recovery of [ $^3\text{H}$ ]-vincristine

From the determination of radioactivity and concentration carried out as described above, it was calculated that approximately 150 - 200  $\mu\text{Ci}$  of [ $^3\text{H}$ ]-vincristine were recovered from 1.5 mCi of crude product after P11 cellulose phosphate column chromatography. The percentage of recovery in respect to the crude labelled product, depended on the initial amount loaded onto the column. The purified [ $^3\text{H}$ ]-vincristine gave a specific radioactivity between 3 - 5 mCi/mM (800 -1500 cpm/pmole).

#### 4.2.4 [ $^3\text{H}$ ]-vincristine-binding assay

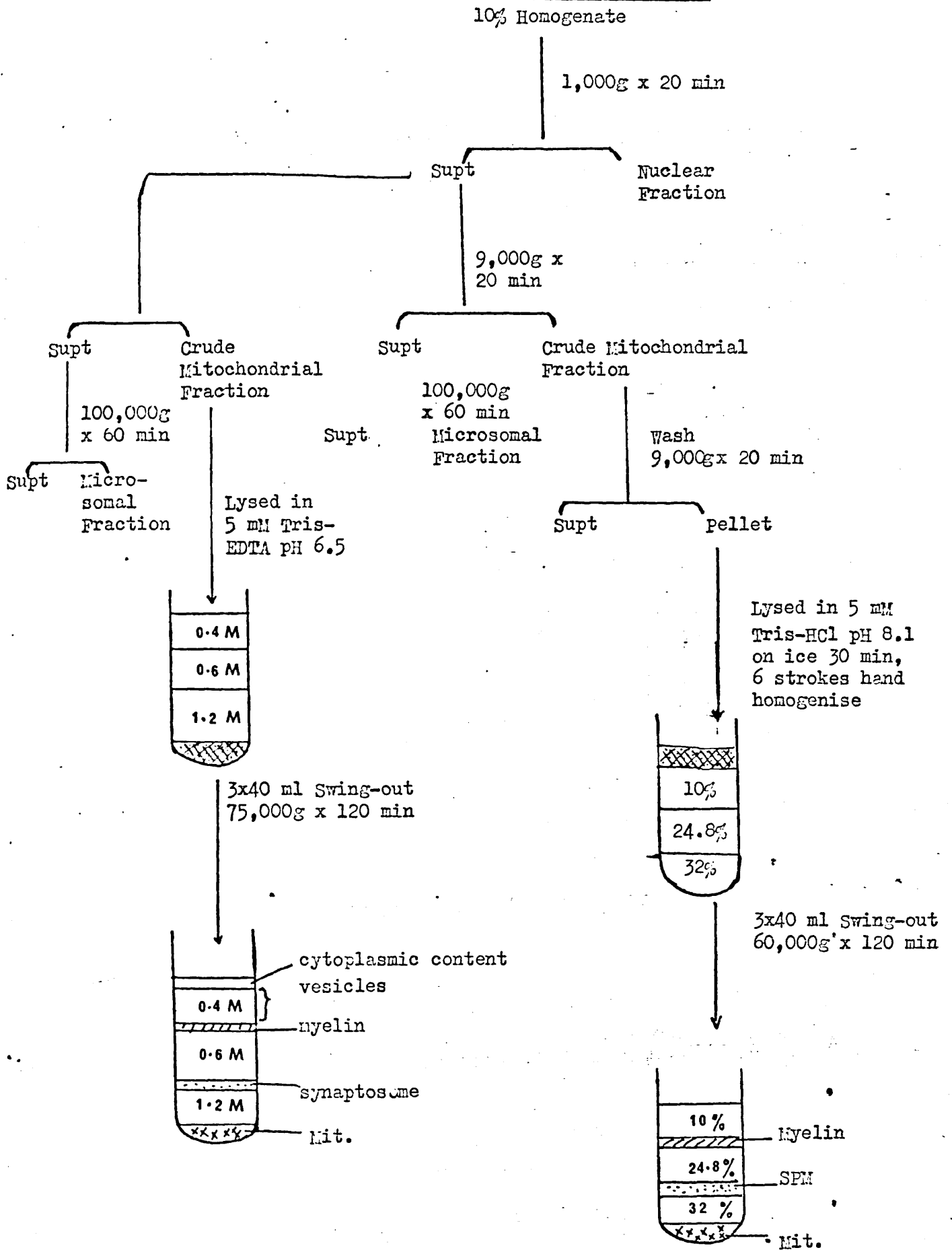
[ $^3\text{H}$ ]-vincristine-binding activity was determined by a filter assay technique similar to that described by Owellen et al. (1972). On the basis of preliminary studies using purified tubulin (obtained by the polymerisation procedure of Shelanski et al., 1973) on the effects of temperature, pH, time of incubation and vincristine concentration on binding activity (for results, see section 4.3.3), the following system was used routinely to assay [ $^3\text{H}$ ]-vincristine binding. Protein (100 - 500  $\mu\text{g}$ ) was incubated with 20  $\mu\text{l}$  of [ $^3\text{H}$ ]-vincristine (6.5  $\mu\text{M}$  final concentration; .1  $\mu\text{Ci}$ )

in 10 mM phosphate ( $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ ) buffer pH 6.8 containing 5 mM- $\text{MgCl}_2$  in a final total volume of 0.3 ml. After incubation for 15 min at  $37^\circ\text{C}$ , the reaction was terminated by chilling on ice and an aliquot of 0.1 ml was transferred onto DEAE impregnated filter disc (Whatman DE 81) which had been previously mounted on the Millipore filtration apparatus and moistened with cold 10 mM-phosphate buffer pH 6.8 containing 5 mM- $\text{MgCl}_2$ . The filter disc was washed three times, each with 5 ml of the same ice-cold buffer under mild suction. Each disc was counted in 5 ml of Bray's scintillant. Duplicate determinations were carried out on each sample and blanks with heat-denatured (5 min at  $100^\circ$ ) proteins were included in the same experiment. Preliminary studies of this assay method showed that three or four washes with 5 ml of buffer each, were necessary to remove free  $\{^3\text{H}\}$ -vincristine from the disc. In all experiments a heat-denatured sample was included as blank. The blank usually contributed not more than 10 % of <sup>the</sup> total counts found for active tubulin preparation.

#### 4.2.5 Subcellular fractionation

In the experiments concerning the subcellular distribution of  $\{^3\text{H}\}$ -vincristine-binding activity, two methods of subcellular fractionation (summarised in Fig. 4.5) were employed. The method of Jones and Matus (1974) was used in particular to obtain purified synaptic plasma membranes, while that of Bradford (1969) (see also DeBellerocche, 1973) was particularly useful for the isolation of fractions enriched in synaptic vesicles, synaptic membranes and synaptic ghosts. In both

( for methods see section 4.2.5.1)



Method of Jones & Natus

Method of Bradford

methods, primary subfractions ("nuclear", "mitochondrial", "microsomal" and "soluble" fractions) were obtained by differential centrifugation, and were further purified by sucrose density gradient centrifugation.

4.2.5.1 Methods of fractionation. (see preparation schemes outlined in Fig.4.5 )

Cerebral cortex tissue from three rat brains was homogenised in 9 volumes of 10 mM-phosphate buffer, pH 6.8, containing 5 mM-MgCl<sub>2</sub>, 0.1 mM-GTP and 10 % sucrose. The homogenate was centrifuged at 1,000 g for 10 min to obtain a crude nuclear fraction, and the supernatant was recentrifuged for 20 min at either 9,000 g (Jones & Matus method) or at 20,000 g (Bradford method). The resulting crude mitochondrial pellet was used as the starting material for further subfractionation by sucrose gradient centrifugation techniques. The remaining supernatant was subjected to high-speed centrifugation at 100,000 g for 1 h to yield the microsomal and soluble primary fractions.

In the method of Jones and Matus (1974) the crude mitochondrial pellet was lysed for 30 min on ice, in cold 5 mM tris-HCl pH 8.1 (2 ml of buffer per g of original tissue), followed by six strokes in a hand-operated homogeniser. The lysate was brought to a concentration of 34 % sucrose by the addition of an appropriate volume of a 48 % solution, and distributed into three 40 ml transparent centrifuge tubes which fitted into the buckets of the 3 x 40 ml swing-out rotor (MSE). The lysate was overlaid with 28.5 % (w/w) sucrose (7.5 - 10 ml), and the upper phase was overlaid with 10 % (w/w) sucrose (7.5 - 10 ml). making a total final volume of 35 ml (see Fig. 4.5). After centrifugation at

60,000 g for 2 h in a MSE Super-speed 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 % sucrose interface, and the mitochondrial fraction was obtained as a pellet. Throughout this method of fractionation, an aliquot of each fraction was taken on which the following assays were performed, on the same day:

<u>Assay</u>	<u>Method reference</u>
1. Protein concentration	(2.7)
2. [ <sup>3</sup> H]-colchicine-binding	(2.3.4)
3. [ <sup>3</sup> H]-vincristine-binding	(4.2.4)
4. Lactic dehydrogenase	(4.2.5.2.a)
5. Fumarase	(4.2.5.2.b)
6. Acetylcholine esterase	(4.2.5.2.c)

In the method of Bradford (1969), the crude mitochondrial pellet was resuspended in cold 5 mM tris-EDTA buffer pH 6.5 (2 ml of buffer per g tissue) and lysed by sucking up and down with a pasteur pipette. The lysate was layered on a three-step density gradient, consisting of 1.2 M, 0.6 M, and 0.4 M sucrose (see Fig. 4.5). Approximately 10 ml of each sucrose layer was required for the 40 ml centrifuge tube. The density gradients were centrifuged at 60,000 g for 2.5 h in the 3 x 40 ml swing-out rotor in an MSE Super-speed 40 centrifuge. Soluble cytoplasmic contents remained in the 10 % sucrose sample layer, while the vesicles were recovered in the 0.4 M sucrose layer. A membrane fraction was obtained in 0.6 M sucrose layer while synaptosome ghosts were recovered from the interface between the 0.6 M and 1.2 M sucrose layers. The mitochondria were collected as a pellet. Samples of each fraction were retained for protein determination,



$^3\text{H}$ -colchicine-binding and  $^3\text{H}$ -vincristine-binding assays

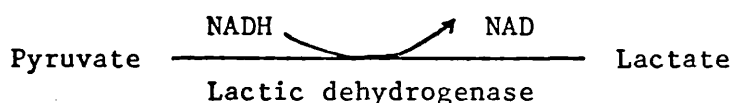
performed on the same day.

#### 4.2.5.2 Assay of marker-enzymes

Convenient enzymic markers were assayed to provide an indication of the purity of each fraction. Lactic dehydrogenase, fumarase and acetyl-choline esterase were used as marker enzymes for the soluble fraction, mitochondria and plasma membrane fractions, respectively (see Han-Ulrich Bergmeyer, 1963; Lai, J. 1975). All reactions were measured at room temperature in a Beckman spectrophotometer, and the rate of reaction was calculated from the linear portion of the tracing obtained with a pen-recorder.

##### (a) Lactic dehydrogenase (LDH) (Clarke & Nicklas, 1970)

This enzyme was assayed by determining the rate of oxidation of excess NADH and pyruvate.

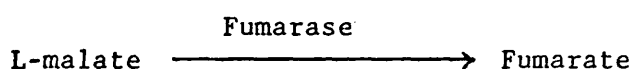


The reaction mixture consisted of 0.2 M  $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$  buffer pH 7.4, 1.5 mM-Na pyruvate (BDH), 0.25 mM-NADH (BDH), 0.05 % triton and tissue extract (100 - 500  $\mu\text{g}$  of protein). The assay was carried out after mixing the following solutions. The reaction was started by the addition of 15  $\mu\text{l}$  of freshly prepared 50 mM-NADH to the sample cuvette which contained 0.3 ml of 0.13 M-Na-phosphate buffer, pH 7.4, 10  $\mu\text{l}$  of 0.5 M-Na-pyruvate adjusted to pH 6.8 with tris, 15  $\mu\text{l}$  of 10 % (v/v) triton X-100 and 25-100  $\mu\text{l}$  of tissue extract, as in the control cuvette. The rate of disappearance of NADH was measured at 340 nm. The rate

of LDH activity was calculated from the molar extinction coefficient of NADH (at  $\lambda$  338 nm,  $e = 6220$ ) and expressed as nmole/min/mg of protein (See Fig. 4.6).

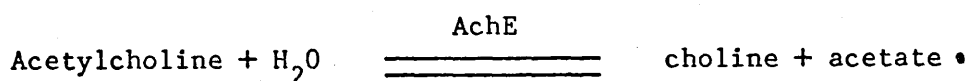
(b) Fumarase (Racker, 1950)

Fumarase activity was measured by following the increase in optical density (O.D.) at 240 nm due to the formation of fumarate from L-malate. Trion X-100 was used to maximise enzyme activity.

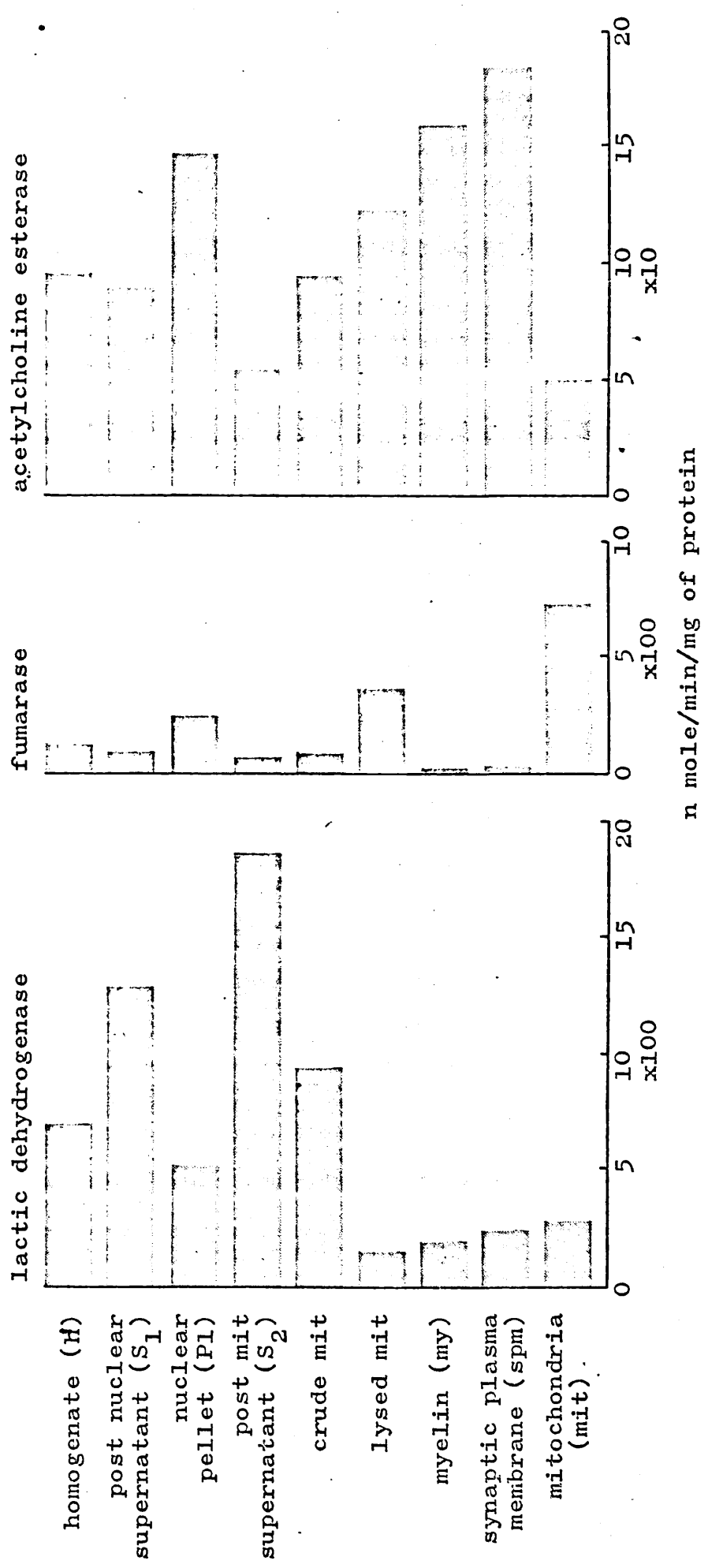


The reaction mixture consisted of 0.2 M Na-phosphate buffer, pH 7.4, 0.1% triton X-100, 30 mM Na-L-malate (Sigma) and tissue extract (100 - 400  $\mu$ g of protein). The assay was carried out after mixing of the following solutions. The reaction was started by the addition of 0.2 ml of a 0.5 M solution of L-malate (adjusted to pH 6.8 with NaOH) to a reaction mixture containing 3 ml 0.2 M Na-phosphate buffer pH 7.4, 30  $\mu$ l of 10% (v/v) triton X-100 and 25 - 100  $\mu$ l of tissue extract. From the molar extinction coefficient of fumarate<sup>at</sup>  $\lambda$  240 nm,  $e = 2.44 \times 10^6$  the rate of fumarate formation was calculated and expressed as nmole/min/mg of protein.

(c) Acetylcholine esterase (AChE; Ellman et al., 1961)



The reaction mixture consisted of 0.1 M Na-phosphate buffer, pH 8.0, 0.4 mM-5,5 dithio-bis-2-nitrobenzoic acid (DTNB), 0.6 mM-acetylthio-choline iodide (Koch Light) and tissue extract containing between 50 - 250  $\mu$ g of protein. Stock solutions were



**Fig.4.6.** Distribution of enzymes in rat brain subcellular fractions prepared by the method of Jones 7 Matus (1974) z(see section 4.2.5. for details)

prepared and reaction was measured as follows.

The increase in absorbance at 416 nm was recorded, after addition of 10-50  $\mu$ l of tissue extract was added to a reaction mixture containing 3 ml of 0.1 M Na-phosphate buffer pH 8.0, 0.12 ml of freshly prepared 10 mM DTNB dissolved in the same buffer, and 30  $\mu$ l of 75 mM-acetylthiocholine iodide. The rate of acetylcholine esterase activity was calculated from the molar extinction coefficient at  $\lambda$  416,  $e = 1.36 \times 10^4$ .

#### 4.2.5.3. Results

It was found that the post-nuclear supernatant (SI) and post mitochondrial supernatant (S2) fractions were particularly enriched in lactic dehydrogenase activity, while the subfractions obtained from the lysed crude mitochondrial pellets (P2) were relatively low in LDH activity (see Fig. 4.6). As might be expected, fumarase activity was exceptionally high in the final mitochondrial pellet (Mit) fraction obtained after sucrose gradient centrifugation, giving twice the activity of the lysed mitochondrial fraction before further fractionation (see Fig. 4.6). The highest values for acetylcholine esterase activity were found in the synaptic plasma membrane (SPM) and myelin (My) fractions, as shown in Fig. 4.6. In addition, polyacrylamide gel electrophoresis of synaptic membrane fractions purified in this way showed identical protein patterns as have been found for similarly prepared synaptic plasma membranes which were morphologically identified by electron microscopy (B. Walter, personal communications; see also Chapter III, section 6).

### 4.3 Results

#### 4.3.1 The binding of vincristine to microtubular protein

In order to confirm the binding of [ $^3\text{H}$ ]-vincristine to microtubular protein, G100 Sephadex chromatography was employed to separate the protein-bound [ $^3\text{H}$ ]-vincristine from the free [ $^3\text{H}$ ]-vincristine.

From Fig. 4.7, it is apparent that tubulin-bound [ $^3\text{H}$ ]-vincristine was completely separated from free [ $^3\text{H}$ ]-vincristine, and that the tubulin-bound [ $^3\text{H}$ ]-colchicine peak coincided with the tubulin-bound [ $^3\text{H}$ ]-vincristine peak. The free [ $^3\text{H}$ ]-vincristine (M.W. 922) was eluted slightly earlier than the free [ $^3\text{H}$ ]-colchicine (M.W. 399), as might be expected.

From these experiments, it is therefore obvious that [ $^3\text{H}$ ]-vincristine bound to tubulin.

#### 4.3.2 Vincristine-induced precipitation of microtubular protein

It has been reported that low concentrations of vinblastine or vincristine can induce the aggregation of tubulin (Weisenberg & Timasheff, 1970; Lagnado & Lyons, 1972; Wilson *et al.*, 1974).

When foetal rabbit brain supernatant obtained by centrifugation of a 10 % homogenate at 100,000 g for 1 h, was incubated with vincristine at a concentration of 400  $\mu\text{M}$  for 20 min, the aggregates formed were pelleted by high-speed centrifugation ( $10^5$  g x 60 min) and analysed by polyacrylamide gel electrophoresis in a phosphate buffered system. Tubulin was found to be the major component (see Fig. 2.6. ). At least three

Fig.4.7. Separation of tubulin-bound [ $^3\text{H}$ ]colchicine and [ $^3\text{H}$ ]vincristine by chromatography on Sephadex G100.

---

Tubulin purified from rat brain by the polymerisation procedure of Shelanski *et. al.* (1973) was incubated with [ $^3\text{H}$ ]vincristine and buffer as described in 4.24. Incubated samples were loaded on a G100 Sephadex column (2.5x20 cm) placed in a refrigerated LKB fraction collector maintained at 4°. The column was eluted with 10 mM-phosphate buffer, pH 6.8, containing 5mM-MgCl<sub>2</sub>. Fractions (1 ml) were collected and aliquots (0.1 ml) of each fraction was counted in 5 ml of Bray's scintillant. The remaining 0.8 ml was used for protein determination by the Lowry method. An identical incubation mixture, except that vincristine was replaced by [ $^3\text{H}$ ]colchicine (2.5  $\mu\text{M}$ ; 0.4  $\mu\text{Ci}$ ), was incubated for 1.5 h at 37°. The reaction was terminated by cooling on ice and the mixture was similarly applied to the same column which had been previously washed with buffer to remove any trace of radioactivity.

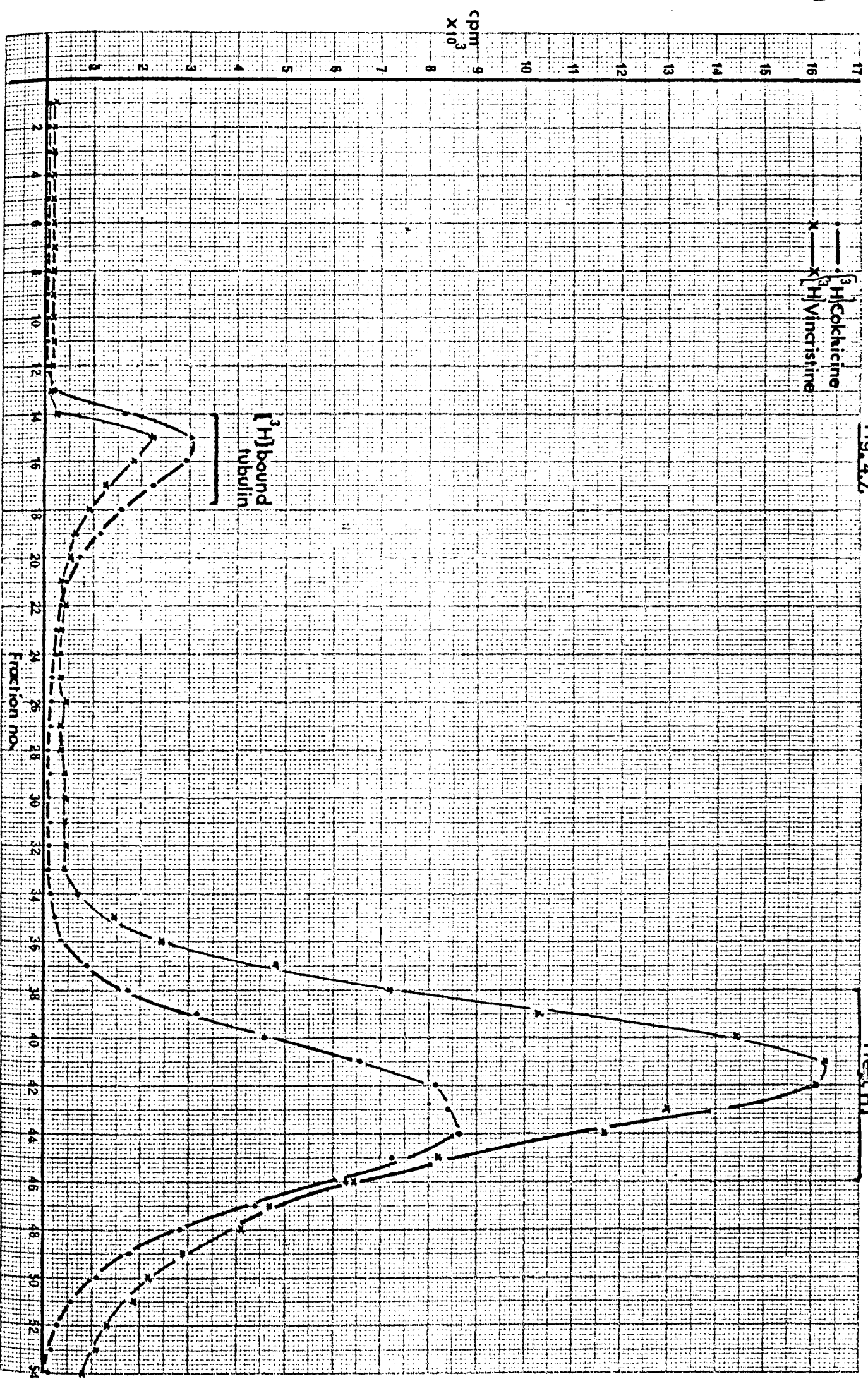


Fig. 4.2

other minor proteins were also detected in this preparation. Further, similar experiments on a supernatant which had been preincubated with [ $^3\text{H}$ ]-colchicine, revealed a  $97 \pm 3\%$  distribution of the protein- $^3\text{H}$ -colchicine was recovered in the pellet fraction, as compared to  $10 \pm 5\%$  of the labelled complex in the control sample without added vincristine (see also Chapter III, 3.2.3. ). These data substantiate earlier observations on the vincristine induced aggregation of tubulin (Lagnado & Lyons, 1972).

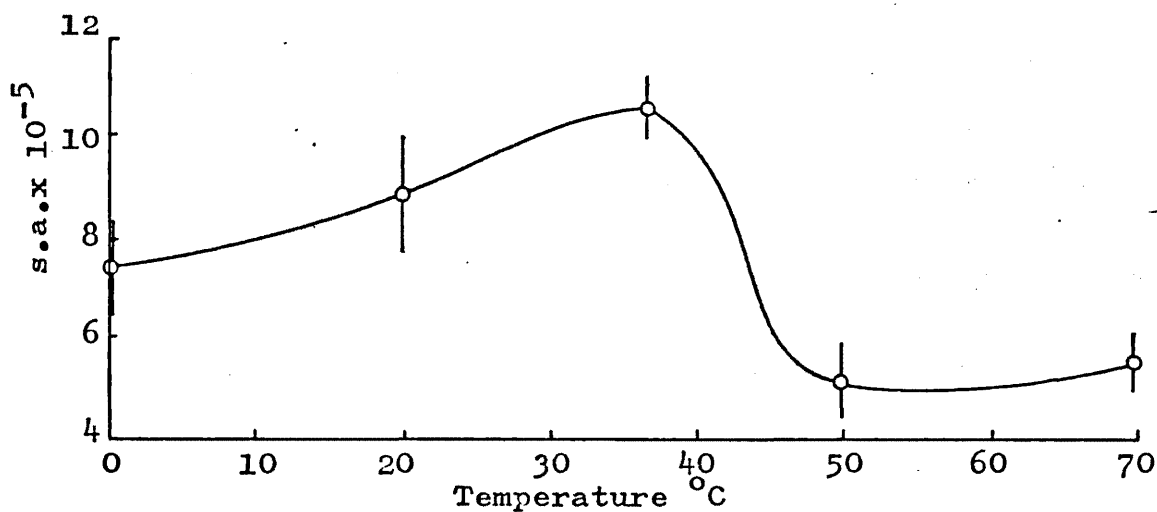
From these data and those obtained by column chromatography (4.3.1), it can be concluded that [ $^3\text{H}$ ]-vincristine is in fact bound to tubulin under the conditions stated.

#### 4.3.3 Characteristics of [ $^3\text{H}$ ] vincristine-binding to purified tubulin

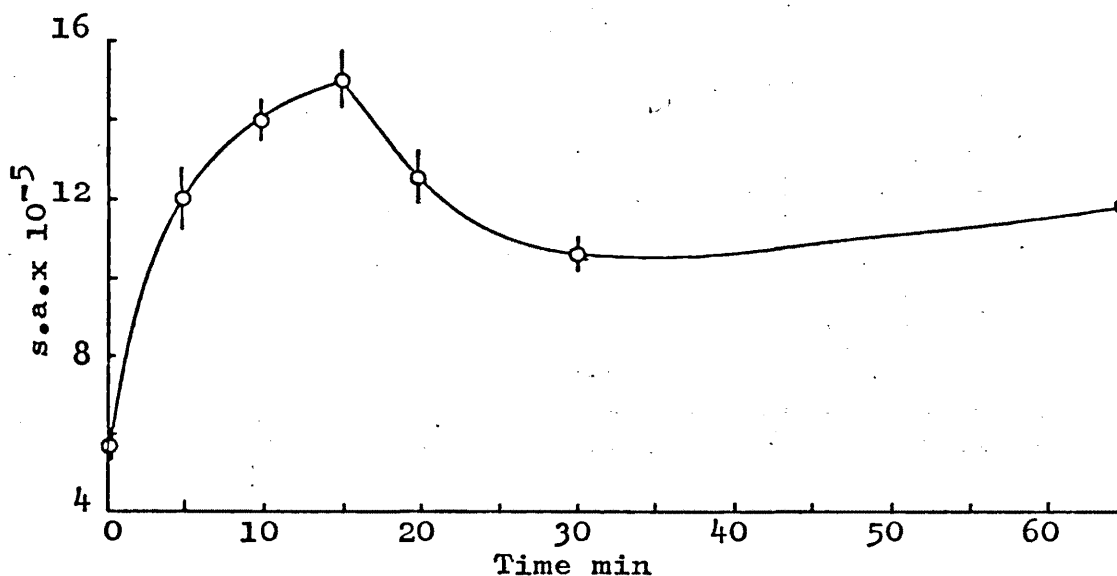
Preliminary investigations using filter disc assay (4.2.4) revealed that rat brain tubulin purified by the method of Shelanski et. al. (1973) exhibited maximum binding activity at  $37^\circ$  and after incubation for 15 min (see Fig. 4.8. a & b). An optimal pH of 6.5 was found for the binding reaction, as shown in Fig. 4.9.c. Under these optimal conditions of assay, the saturation concentration was found to be  $6.5 \mu\text{M}$ , as shown in Fig. 4.9. A double reciprocal plot of these data (see inset, Fig. 4.9.) gave a straight line, from which an apparent  $K_d$  of  $8.3 \times 10^{-6} \text{M}$  was obtained for the binding reaction.



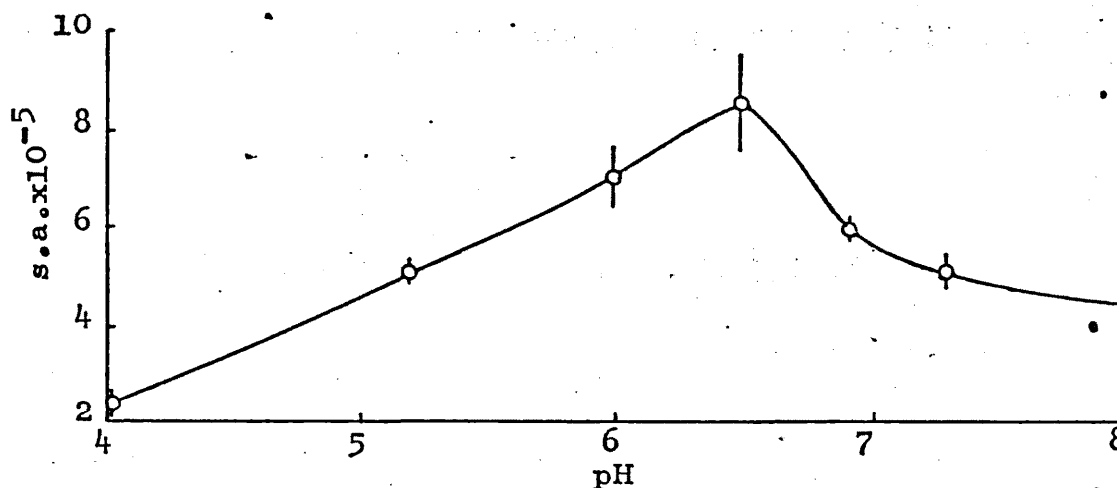
(a)



(b)

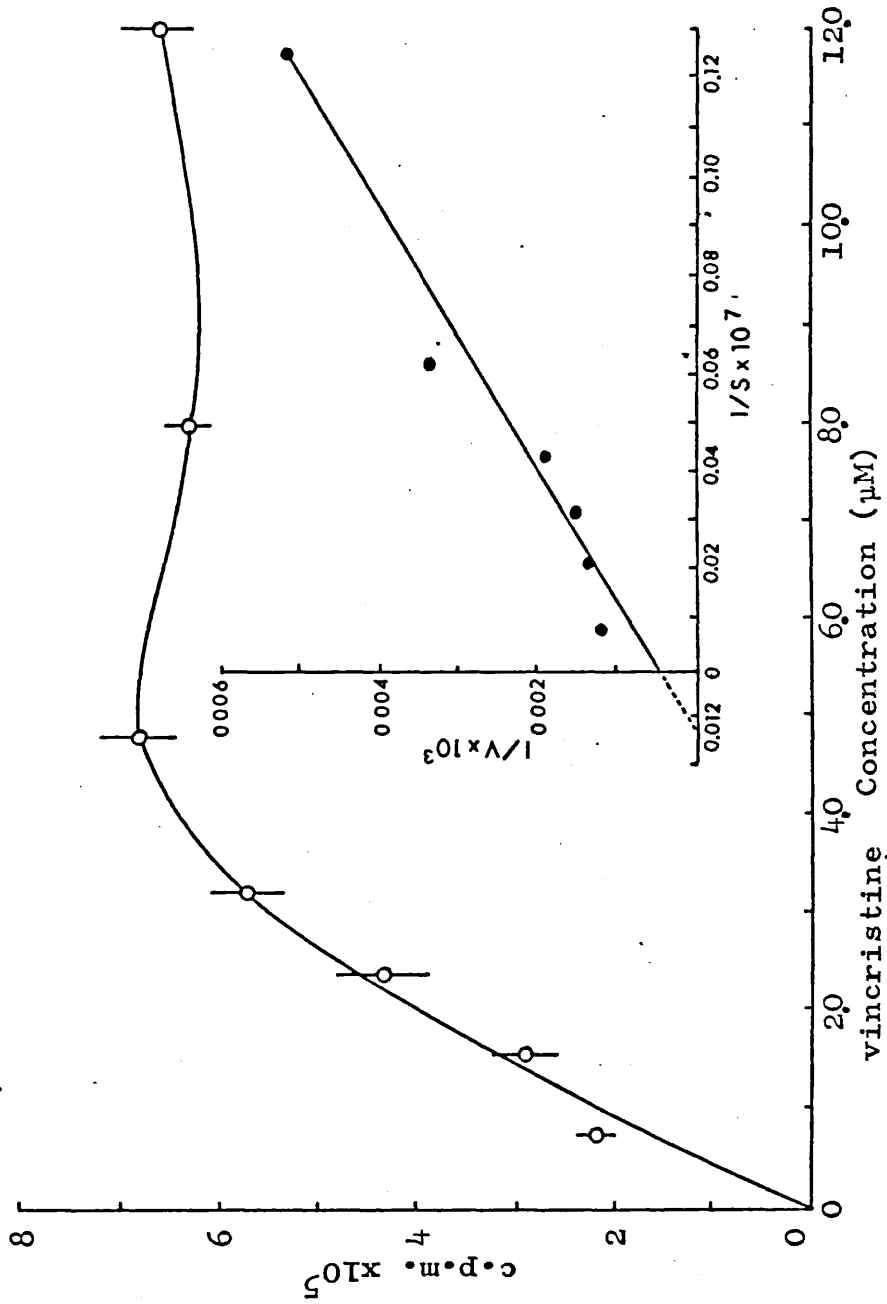


(c)



Rat brain tubulin was incubated with  $6.5\mu\text{M}$  [ $^3\text{H}$ ]vincristine for 15 min (a,b) at 37° (b,c) and the protein-bound [ $^3\text{H}$ ] was determined by the DE 81 filter disc assay (for details, see 4.2.4.) (S.A. = bound cpm/mg protein).

**Fig.4.9.** Effects of vincristine concentration on the binding of [ $^3\text{H}$ ]vincristine to rat brain tubulin.



Tubulin was incubated in the presence of vincristine at the concentrations indicated at  $37^\circ$  for 15 min prior to assay of the bound [ $^3\text{H}$ ] by the DE 81 filter assay method.

#### 4.3.4 The effects of colchicine and GTP on the binding of [<sup>3</sup>H]-vincristine to tubulin

It was of some interest to know whether colchicine or GTP might interfere with the binding of [<sup>3</sup>H]-vincristine to tubulin.

Experiments using the filter disc assay repeatedly showed that colchicine at a concentration of 100  $\mu$ M inhibited the binding of [<sup>3</sup>H]-vincristine to purified tubulin by 10 %. At a concentration of (10  $\mu$ M), colchicine caused a 30 - 40 % increase in vincristine-binding activity, whereas lower concentrations (1  $\mu$ M and 0.1  $\mu$ M) tested had negligible effects. GTP had no apparent effect on [<sup>3</sup>H]-vincristine-binding over a wide range of concentrations tested (0.1 - 100  $\mu$ M).

These data may indicate that vincristine is bound to specific sites on tubulin which are apparently distinct from the colchicine or GTP-binding sites.

#### 4.3.5 Subcellular distribution of [<sup>3</sup>H] vincristine and [<sup>3</sup>H] colchicine binding receptors

Early work by Lagnado et al. (1971) reported that a significant proportion of the colchicine-binding activity present in rat brain homogenates could be recovered in a fraction enriched in synaptic membranes. Very recent evidence by Lagnado et al. (1975) further substantiates the view that tubulin-like proteins are present in synaptic membranes purified from pig cerebellum by the method of Jones & Matus (1974) (see 4.2.5.1). In preliminary experiments, the [<sup>3</sup>H] vincristine-binding activity of such membrane preparations was found to be quite high giving specific activities (bound cpm/mg protein) about half of those for tubulin purified by the method of microtubule assembly in vitro. In the light of these findings

the subcellular distribution of vincristine-binding receptors was further investigated. The [ $^3\text{H}$ ]-colchicine-binding activity, which is used as an indication of the amount of tubulin present, was also determined alongside with the estimation of [ $^3\text{H}$ ]-vincristine-binding activity in each subcellular subfraction. A comparison of the distribution of binding activity for two drugs should give some information as to the specificity of the vincristine- and colchicine-binding receptors.

As shown in Fig. 4.10.a, about 68 % of the protein in the homogenate was recovered in the nuclear fraction (N), which accounted for 56 % and 81 % of the recovered binding activity for colchicine and vincristine, respectively. However, it should be emphasized that the crude nuclear fraction assayed was heavily contaminated with non-nuclear particulates, as well as containing trapped soluble components. Further purification of this fraction would therefore be necessary to determine the extent to which binding activity was associated with nuclei. The crude mitochondrial fraction (Mit) contained 8 % of total protein recovered, and 6 % and 8 % respectively of the recovered colchicine- and vincristine-binding activities. Among the four primary fractions, the soluble fraction was enriched in colchicine-binding activity, but gave the lowest values for vincristine-binding.

These results, summarised in Fig. 4.10.a, indicate that a high proportion of the total tubulin, active in binding colchicine, remains in the soluble fraction. On the other hand, the bulk of vincristine-binding receptors are associated with the particulate fractions, i.e. the nuclear, mitochondrial and microsomal

Fig. 4.10. Subcellular distribution of colchicine and vincristine binding activities in rat brain homogenates.

---

Subcellular fractionation was carried out by the method of Jones & Matus (1974). [ $^3\text{H}$ ] colchicine and [ $^3\text{H}$ ] vincristine binding were assayed as described in section 2.3.4. and 4.2.4, respectively.

Fig. 4.11. Subcellular distribution of colchicine and vincristine binding activities in rat brain homogenates

---

Subcellular fractionation was carried out by the method of Bradford (1969). [ $^3\text{H}$ ] colchicine and [ $^3\text{H}$ ] vincristine binding were assayed as described in section 2.3.4. and 4.2.4, respectively.

---

#### Abbreviations

N= nuclear  
 Mit =crude mitochondrial  
 Mc =microsomal  
 S =soluble  
 Sol =soluble  
 My =myelin  
 Spm =synaptic plasma membrane  
 Mit--mitochoncrial  
 Ves =synaptic vesicles  
 Mb ='light' membranes  
 syn =synaptosome 'ghost'

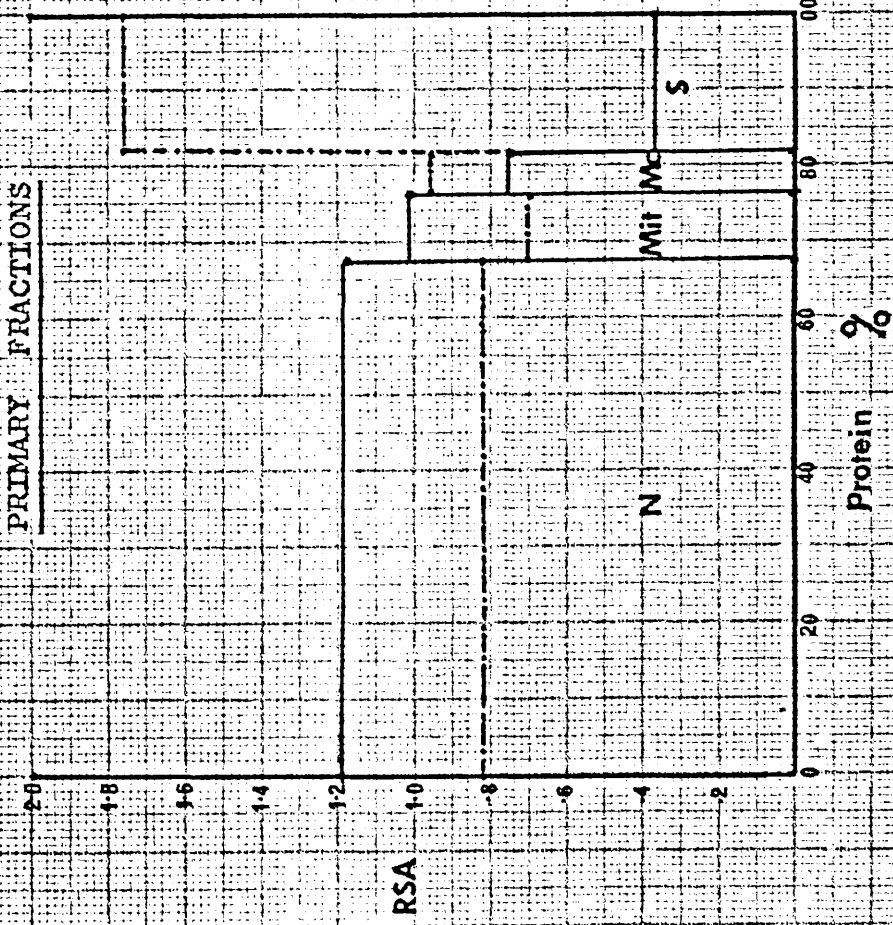
RSA =  $\frac{\% \text{ recovered bound cpm}}{\% \text{ recovered protein}}$

[<sup>3</sup>H] Vincristine  
 [---] [<sup>3</sup>H] Colchicine

Fig. 4.10.

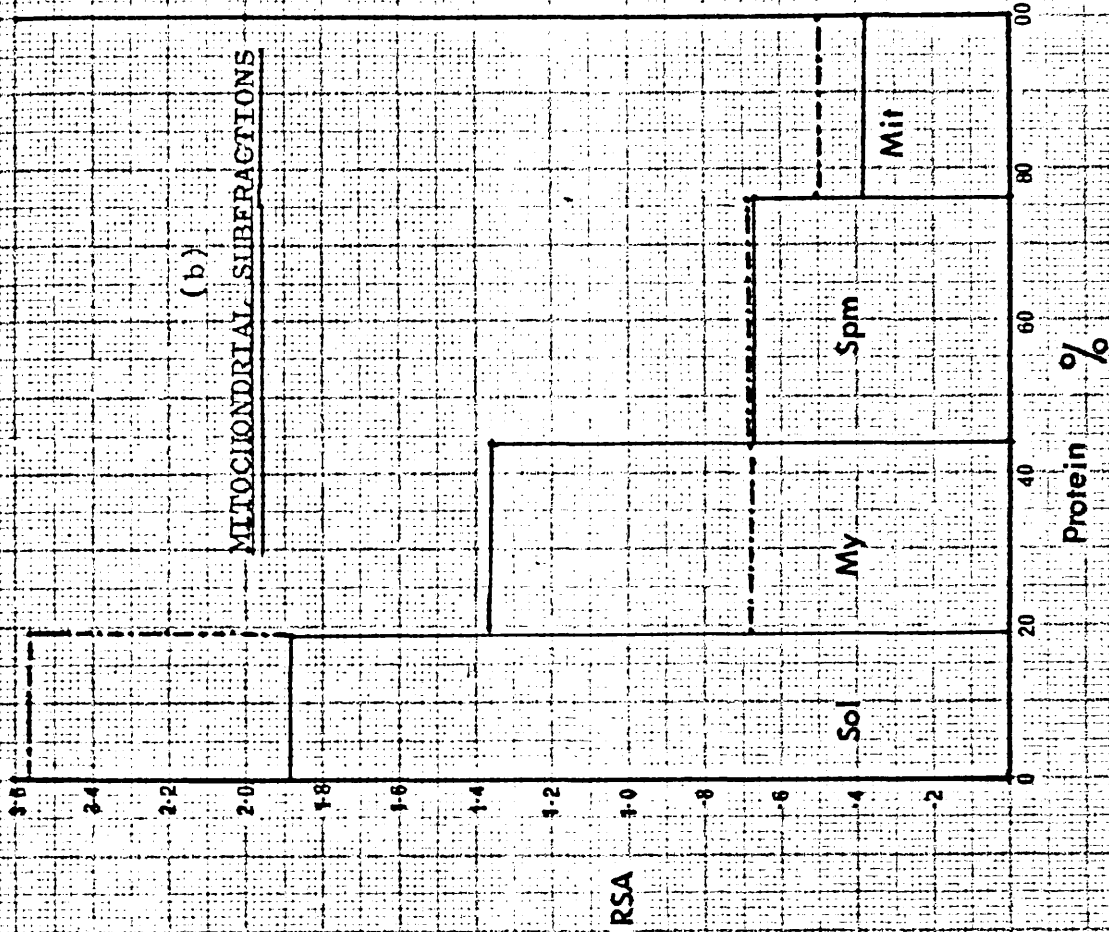
PRIMARY FRACTIONS

(a)



MITOCHONDRIAL SUBFRACTIONS

(b)

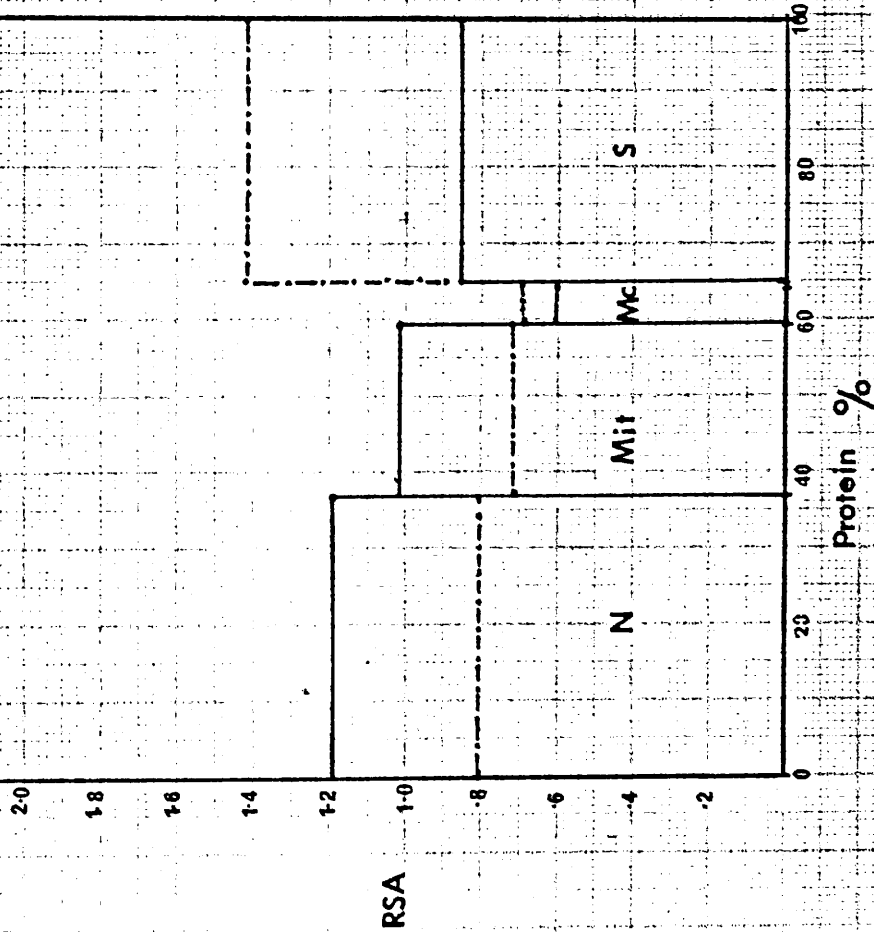


— <sup>3</sup>H Vincristine  
 - - - <sup>3</sup>H Colchicine

Fig.4.11.

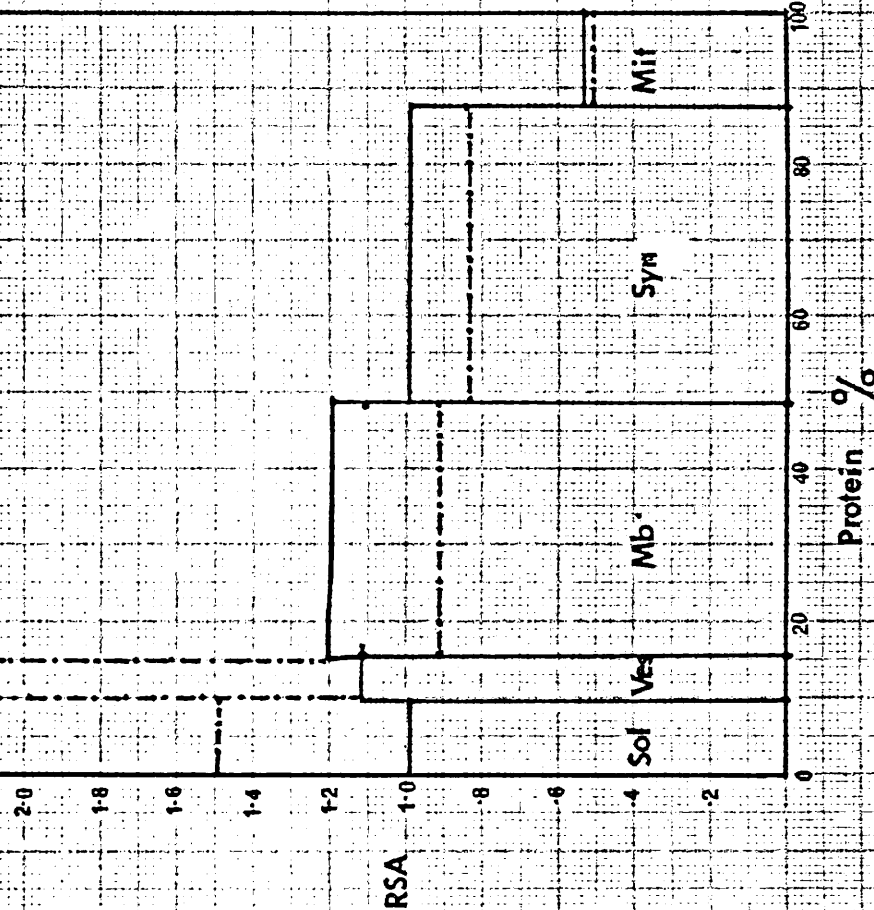
(a)

PRIMARY FRACTIONS



(b)

MITOCHONDRIAL SUBFRACTIONS



fractions, in that order of decreasing relative specific activity (RSA).

Further fractionation of the lysed crude mitochondrial fraction by density gradient centrifugation (as shown in Fig. 4.5; method of Jones and Matus, 1974), showed that the material recovered in the soluble subfraction (Sol) (10 % sucrose layer) gave the highest values in terms of RSA or percentage distribution of colchicine and vincristine-binding activities. Besides the 10 % sucrose layer, the myelin fraction also contained high vincristine-binding activity although the colchicine-binding activity was relatively low (see Fig. 4.10.b). The nature of materials in the soluble fraction prepared by this method could not be ascertained unequivocally, since any synaptosomal membrane material (e.g. synaptic vesicles) lighter than myelin could float into this layer. Therefore a more elaborate method of subfractionation described by Bradford (see Fig. 4.5) was carried out in order to better identify the subcellular components responsible for the binding of colchicine and vincristine. Primary fractions obtained by the method of Bradford showed considerable differences in the distribution of protein from that found with the method of Jones and Matus (see Fig. 4.11a): Less protein was recovered in the nuclear fraction, and approximately twice the amount of protein was recovered in the mitochondrial and soluble fraction. Nevertheless, with the exception of the values found for vincristine-binding activity in the soluble fraction, the R.S.A. values for colchicine and vincristine-binding in the primary fractions showed rather similar patterns using either subcellular fractionation procedure. The variation in the recovery of protein in the various fractions is probably related to differences in time and speed of centrifugation used in the two methods.



Subfractionation of the lysed mitochondrial fraction on density gradient centrifugation by the method of Bradford clearly showed that the synaptic vesicle and soluble fractions were enriched in colchicine-binding activity (see Fig. 4.11.b). However, the light membrane fraction recovered from the 0.6 M sucrose layer had the highest vincristine-binding activity, giving RSA value of 1.2, while the vesicle fraction (Ves), which was enriched in colchicine-binding activity, gave slightly lower values (RSA = 1.12). The soluble synaptic membranes ("ghosts") and mitochondrial fractions were relatively poor in vincristine-binding activity, having RSA of 1 or less.

These findings support the evidence obtained using the Jones and Matus fractionation procedure (see above & Fig. 10.b) that membranes and synaptic vesicles are relatively enriched in vincristine-binding receptors.

#### 4.4 Discussion

From the data presented it is apparent that vincristine binds to purified tubulin in a time, temperature, pH and concentration-dependent manner. The lack of effect of GTP at the concentration between 0.1 - 10  $\mu$ M and colchicine (0.1 and 1  $\mu$ M) on the vincristine-binding activity of tubulin suggests that the vincristine-binding sites are distinct from the colchicine and GTP-binding sites. The reason for an increase of 30 % vincristine-binding activity due to the addition of 10  $\mu$ M colchicine is not known.

Experiments on subcellular distribution of vincristine-binding receptors indicate that besides the crude nuclear fraction the membrane subfraction and the synaptic vesicles

fraction are relatively enriched in vincristine-binding receptors. This is particularly interesting in view of the recent findings by Hanbauer et al. (1974) that when injected in vivo vinblastine caused a decrease in number of fluorescent adrenergic terminals in rat atria. They also showed that vinblastine either injected in vivo to rat or added to rat heart atria in vitro induced prolonged inability to take up [ $^3\text{H}$ ]-noradrenaline, an effect being interpreted as the destruction of adrenergic nerve terminals. Whether this effect occurs solely in adrenergic nerve terminals remains to be investigated. However, in subcellular fractionation, heterogeneous membranes and vesicles preparations are obtained and until the specificity of vinca alkaloids to the type of nerve terminals is known, these binding data should be interpreted with caution. Nevertheless, our recent identification of tubulin-like protein in the synaptic membrane isolated from pig brain cerebellum (Lagnado et al., 1975), lends support to the interpretation of Hanbauer et al. (1974) that vinblastine may disrupt nerve terminals by acting on a membrane-bound form of tubulin. The possible role of tubulin in controlling the integrity and function of synaptic membranes will be discussed in the final chapter.

Chapter V            The effects of drugs on the binding properties of  
tubulin

5.1            Introduction

5.2            Effects of drugs on colchicine-binding activity

5.2.1          Preparation of drugs

5.2.2          Comparison of the effects of vincristine and reserpine  
on colchicine-binding activity using different methods  
of assay

5.2.3          The effects of Vinca alkaloids and related compounds  
on the properties of foetal rabbit brain tubulin

5.3            Effects of drugs on GTP-binding activity

5.4            Effects of drugs on the aging of colchicine-binding  
activity

5.5            Effects of reserpine on rat brain after in vivo  
administration

5.5.1          Effects of in vivo administration of reserpine on  
colchicine-binding activity in soluble and  
particulate fractions of rat brain

5.5.2          The uptake and binding of colchicine in cerebral  
cortex slices from reserpinised rat

5.6            Discussion

Chapter V      The effects of drugs on the binding properties of tubulin

5.1      Introduction

Early observations that vincristine increased the colchicine-binding activity of foetal rabbit brain extracts in a concentration-dependent manner (see Chapter III, 3,2,3) and that it also promoted the binding of GTP to purified tubulin, motivated further investigation into the effects of Vinca alkaloids and other structurally related compounds on the various binding activities of tubulin. Competition experiments were carried out in the hope that more information concerning the structural specificity of colchicine and Vinca alkaloids binding sites might be obtained to explain the mode of action of antimitotic drugs on microtubules. In this work, labelled colchicine and vincristine were used as investigative tools to study the biochemical and functional properties of microtubules. This approach was also extended to an in vivo system in which the relative distribution of colchicine-binding protein in the soluble and particulate fractions obtained from the brain of reserpinised animals was compared to that of control animals. Additional experiments on colchicine uptake in brain slices of reserpinised rats will also be reported.

5.2      Effect of drugs on colchicine-binding activity

In agreement with the earlier observations that vincristine and vinblastine increased the colchicine-binding activity of crude tissue extracts, it was found that they exerted the same

activating effect on purified tubulin (see later sections).

This effect was further investigated using other compounds bearing structural similarities to the Vinca alkaloids. The compounds tested fall into two main categories; they are either indole alkaloids or indole amines.

#### 5.2.1 Preparation of drugs

Drugs were normally dissolved in deionised distilled water pH 6.5 in a concentration 10 times higher than the concentration required in the incubation mixture. (On addition of the drug to the buffer incubation mixture, the pH should remain unchanged in respect to the P-Mg buffer, pH 6.8). In the case of the more acidic drug solutions such as harmine HCl or yohimbine HCl, sodium hydroxide was added to neutralise them before use. Drugs which were sparingly soluble in distilled water were dissolved in the appropriate organic solvents and the pH of the solutions was adjusted accordingly. Reserpine was dissolved in a citric acid-propanol-propylene glycol solvent system and adjusted to pH 6.5 with sodium hydroxide prior to addition to the incubation mixture. For preparation of a 0.1 M-reserpine solution, ca. 6 mg of reserpine (Roche) was dissolved in 0.3 ml of 1 M-citric acid and 0.6 ml of propanol. To this solution 6.6 ml of propylene glycol was added to stabilise the drug, followed by an addition of 2.5 ml of 0.1 M-Na-phosphate buffer, pH 6.8. The pH was then adjusted to 6.5 with NaOH and the solution stored in a brown bottle. Melatonin and 6-methoxy-indole (Koch-Light) were initially dissolved in a small amount of absolute alcohol and diluted with distilled water to give a final alcohol concentration of 4 % and 10 %, respectively. In all the tests

Table 5.1

Effects of vincristine and reserpine on colchicine-binding activity

<u>Addition</u>	<u>Assay Method</u>			
	<u>G100 Sephadex chromatography</u>	<u>DE 81 filter disc assay</u>	<u>Millipore filtration assay</u>	
	<u>S.A. x 10<sup>3</sup> cpm</u>	<u>%</u>	<u>S.A. x 10<sup>3</sup> cpm</u>	<u>%</u>
Buffer control	2.45	100	1.96	100
100 $\mu$ M VC	4.51	183	3.92	200
Reserpine carrier control	1.76	100	1.48	100
100 $\mu$ M Reserpine	1.20	68	1.07	72
			3.08	100
			5.85	190
			2.42	100
			1.82	75

Tubulin was incubated with [<sup>3</sup>H]colchicine in the presence of 100  $\mu$ M-vincristine or 100  $\mu$ M-reserpine. Control incubation mixtures contained an equivalent volume of the appropriate carrier solution. The effect of each drug is expressed as a percent of its appropriate control value (for details see text section 5.2.2).

of these drugs the same volume and concentration of the carrier of each drug was included in controls. All drugs were added to an incubation mixture containing purified tubulin and P-Mg buffer, pH 6.8, before incubation with [ $^3\text{H}$ ]colchicine for 1.5 h at 37°. Colchicine-binding activity was assayed by the DE 81 filter disc assay as described in Chapter II, 2.3.4.

#### 5.2.2 Comparison of the effects of vincristine and reserpine on colchicine-binding activity using different methods of assay

It was initially thought that the increase of colchicine-binding activity of purified tubulin due to the addition of vincristine or the decrease of activity in the presence of reserpine might be an artefact of the filter disc assay employed. The effects of these two drugs were, therefore, examined using the three assay methods previously studied (see Chapter II, 2.3.5).

Tubulin, purified by the batchwise method, was incubated for 90 min at 37° with 2.5  $\mu\text{M}$  [ $^3\text{H}$ ]colchicine in the presence of 100  $\mu\text{M}$  vincristine or 100  $\mu\text{M}$ -reserpine; controls were run in the presence of the same volume of buffer or carrier. The reaction was stopped by cooling on ice and samples were assayed by G100 Sephadex chromatography (2.3.2), the DE 81 filter disc method (2.3.4) and the Millipore filtration method (2.4.2). The data summarised in Table 5.1 shows that with all three assay methods, vincristine (100  $\mu\text{M}$ ) increased colchicine-binding activity by 80 - 100 % of the control value. In contrast, reserpine consistently inhibited the colchicine-binding activity by about 30 %.

The increase and decrease of colchicine-binding activity obtained with vincristine and reserpine are therefore due to the drugs themselves and not to artefacts of the assay products.

Table 5.2

The effects of drugs on the [ $^3\text{H}$ ] colchicine and [ $^3\text{H}$ ] GTP binding activities of foetal rabbit brain tubulin

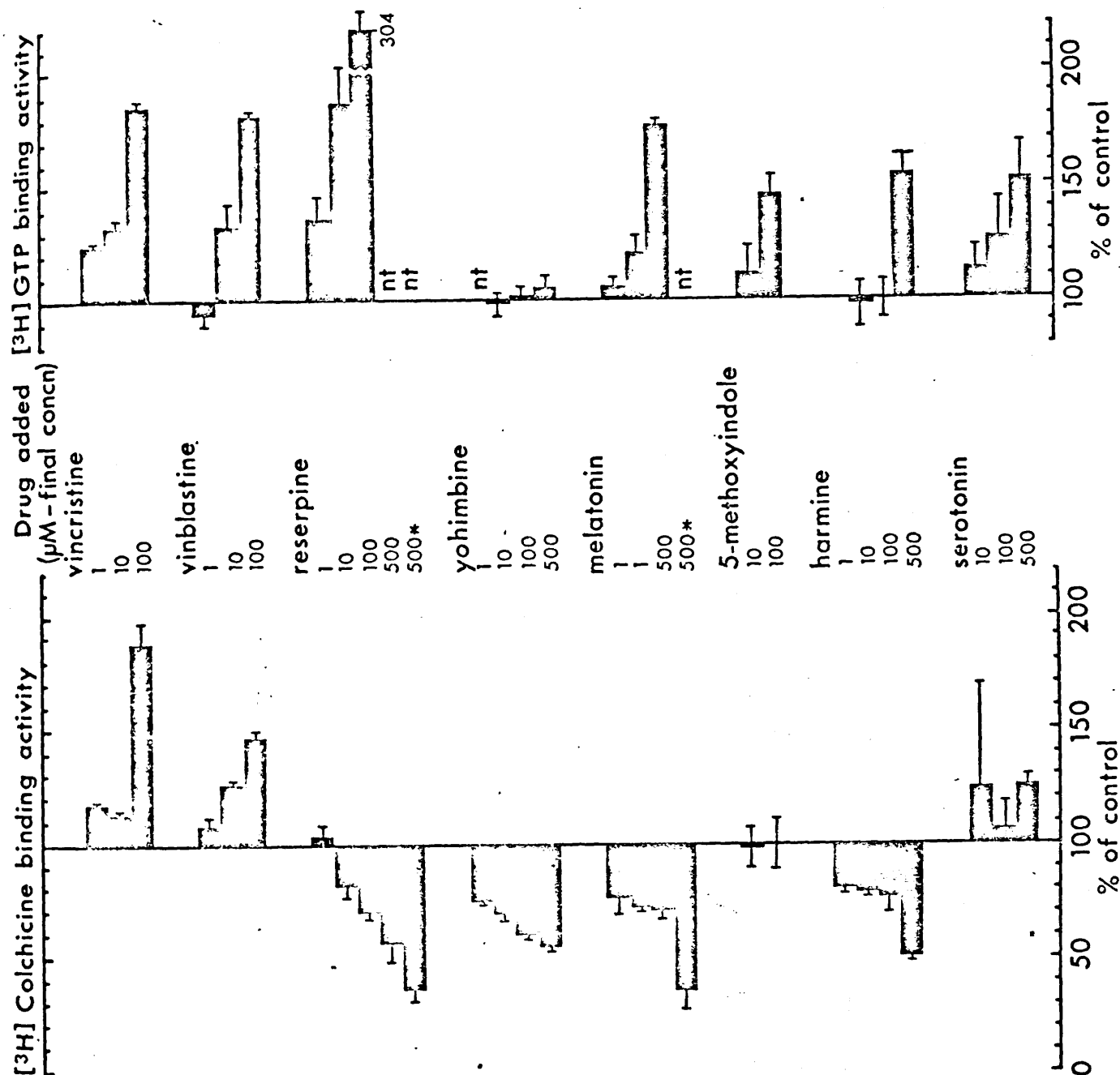
Drug added	final concn. ( $\mu\text{M}$ )	% of control binding activity	
		Colchicine	GTP
Vincristine	1	118 $\pm$ 2	124 $\pm$ 1
	10	114 $\pm$ 2	132 $\pm$ 3
	100	187 $\pm$ 9	185 $\pm$ 3
Vinblastine	1	109 $\pm$ 4	94 $\pm$ 5
	10	127 $\pm$ 2	133 $\pm$ 10
	100	148 $\pm$ 3	181 $\pm$ 2
Reserpine	1	104 $\pm$ 5	136 $\pm$ 10
	10	82 $\pm$ 5	171 $\pm$ 16
	100	70 $\pm$ 3	304 $\pm$ 8
	500	56 $\pm$ 8	n.t.
Yohimbine	1	85 $\pm$ 1	n.t.
	10	80 $\pm$ 3	99 $\pm$ 5
	100	70 $\pm$ 2	102 $\pm$ 4
	500	56 $\pm$ 8	105 $\pm$ 5
Melatonin	10	75 $\pm$ 7	106 $\pm$ 4
	100	71 $\pm$ 2	121 $\pm$ 7
	500	70 $\pm$ 4	176 $\pm$ 2
6-Methoxyindole	10	99 $\pm$ 8	112 $\pm$ 12
	100	100 $\pm$ 11	146 $\pm$ 9
Harmine	1	80 $\pm$ 2	n.t.
	10	78 $\pm$ 2	98 $\pm$ 10
	100	76 $\pm$ 6	100 $\pm$ 8
	500	50 $\pm$ 2	163 $\pm$ 4
Serotonin	10	125 $\pm$ 45	113 $\pm$ 10
	100	107 $\pm$ 12	127 $\pm$ 17
	500	126 $\pm$ 5	153 $\pm$ 16

Tubulin was purified by the batchwise procedure and binding assays were performed at 37 $^{\circ}$  (colchicine) or 4 $^{\circ}$  (GTP) as described in Methods section. Results given are based on at least 3 separate experiments and are expressed as a percentage  $\pm$  S.D. of values obtained in absence of added drug.



Fig.5.1.

Effects of indole alkaloids and related compounds on the colchicine- and GTP-binding activities of foetal rabbit brain tubulin.



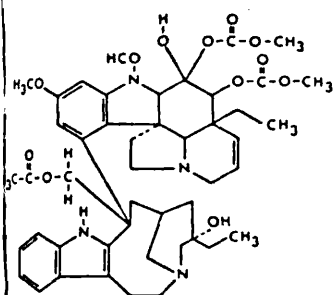
For preparation of tubulin and binding assay methods see text, Chapter II, 2.2. and 2.3.4. Chromatographically purified tubulin was used in all experiments except where indicated by an asterisk (\*), when microtubular subunit protein was derived from reassembled microtubules (see the text, 2.2.3.). Results shown represent the means from four to eight; separate experiments  $\pm$  S.E.M. (thin bar).

nt = not tested.

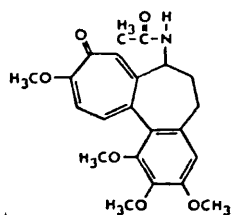
Fig. 5.2.

Structural formulae of indole compounds used  
in this work.

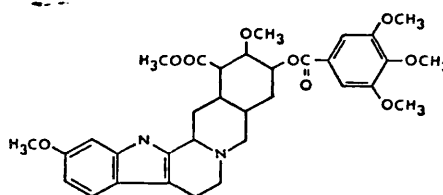
Fig.5.2.



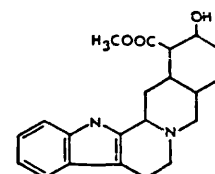
Vincristine



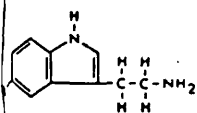
Colchicine



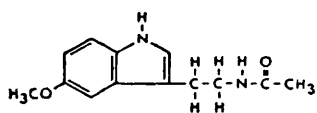
Reserpine



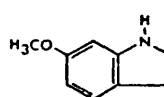
Yohimbine



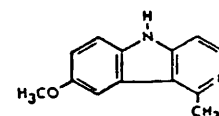
Serotonin



Melatonin



6-Methoxyindole



Harmine

5.2.3 The effects of Vinca alkaloids and related compounds  
on the properties of foetal rabbit brain tubulin

As shown in Table 5.2 & Fig. 5.1, the antimitotic alkaloids vincristine and, to a lesser extent, vinblastine increased the colchicine-binding activity of tubulin isolated from foetal rabbit brain in a concentration- dependent manner in the range tested ( 1 - 100  $\mu\text{M}$  ). This effect is apparently due to the stabilisation by Vinca alkaloids of colchicine-binding sites against thermal inactivation (Wilson, 1970; see also section 5.4). In addition at concentrations in excess of 500  $\mu\text{M}$ , both drugs induced a selective aggregation of tubulin present in crude soluble extracts of foetal or adult rat brain; the precipitates obtained after high-speed centrifugation contained over 95 % of colchicine-binding activity (see 4.3.2).

In marked contrast to the results obtained with Vinca alkaloids, it was found in preliminary experiments that similar concentrations of the three psychotropic indole alkaloids, reserpine, yohimbine and harmine, and of the indole amine, melatonin (for chemical structures see Fig. 5.2) consistently inhibited the colchicine-binding activity of both soluble and particulate extracts of foetal rabbit brain by 30 - 50 %. The effects of these drugs on the colchicine-binding activity of tubulin purified by the batchwise procedure which are summarised in Table 5.2, confirmed the earlier observations concerning the effects of these drugs on crude tissue extracts. In each case, a maximum inhibition of about 60 % was seen at a drug concentration of 500  $\mu\text{M}$ . At concentrations between 100 and 500  $\mu\text{M}$ , reserpine and melatonin increased the turbidity of protein-containing incubation mixtures, as was

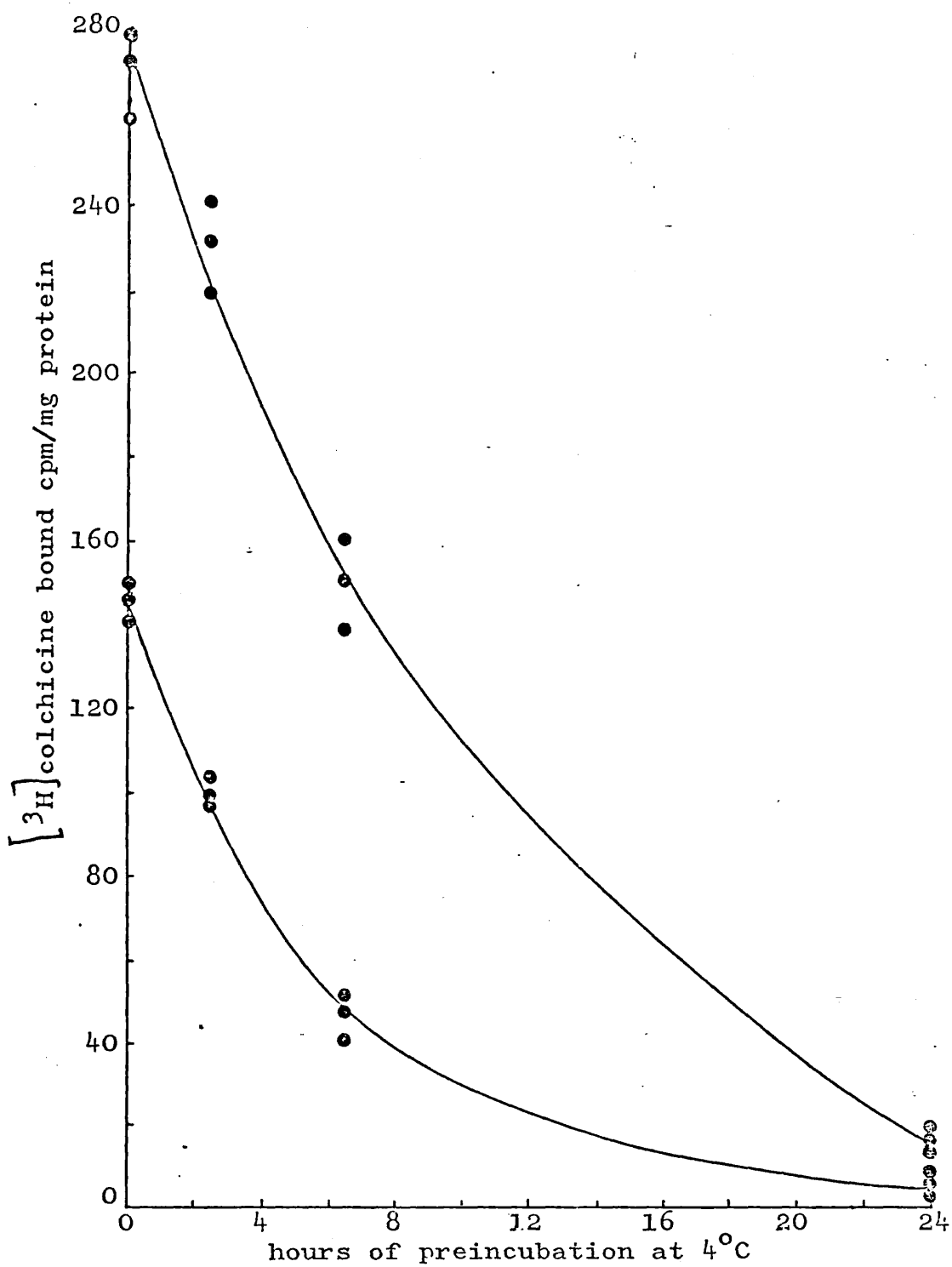
observed with Vinca alkaloids. Serotonin seemed to cause a slight activation but with relatively high statistical variability. Various other compounds tested, such as 6-methoxyindole, tryptamine 5-methoxytryptamine, tetrabenazine, trimethoxybenzoic acid, amphetamine and D-lysergic acid were found to have no effect on the colchicine-binding activity.

### 5.3 Effects of drugs on GTP-binding activity

The observation that some drugs affected the colchicine-binding activity of tubulin led to the investigation of their effects on the GTP-binding activity of the protein as measured by a Millipore filtration assay (for method see Chapter II, 2.4.2) and under the conditions described in 2.4.1. All the drugs, with the exception of yohimbine increased the GTP-binding activity of tubulin in a concentration-dependent manner (see Table 5.2 & Fig. 5.1). Vincristine and vinblastine at concentrations of  $10\ \mu\text{M}$  and  $100\ \mu\text{M}$  increased the GTP-binding activity by 30 % and 80 %, respectively. Reserpine, in contrast to its inhibitory effect on colchicine-binding activity, was found to be most potent in increasing GTP-binding activity; at a concentration of  $100\ \mu\text{M}$ , it caused an increase of approximately 300 %. Melatonin, 5-mthoxyindole and serotonin were less potent while harmine increased the binding activity only  $500\ \mu\text{M}$ . It was interesting to note that colchicine, between the concentrations of 1 -  $100\ \mu\text{M}$ , had no significant effect on the GTP-binding activity (not shown in Table).

Since yohimbine, the only drug tested in which the indole moiety lacked a methoxy group, was without effect on GTP-binding

Fig.5.3. Effects of vincristine ( $100\ \mu\text{M}$ ) on the aging at  $4^\circ$  of colchicine-binding activity of chromatographically purified tubulin



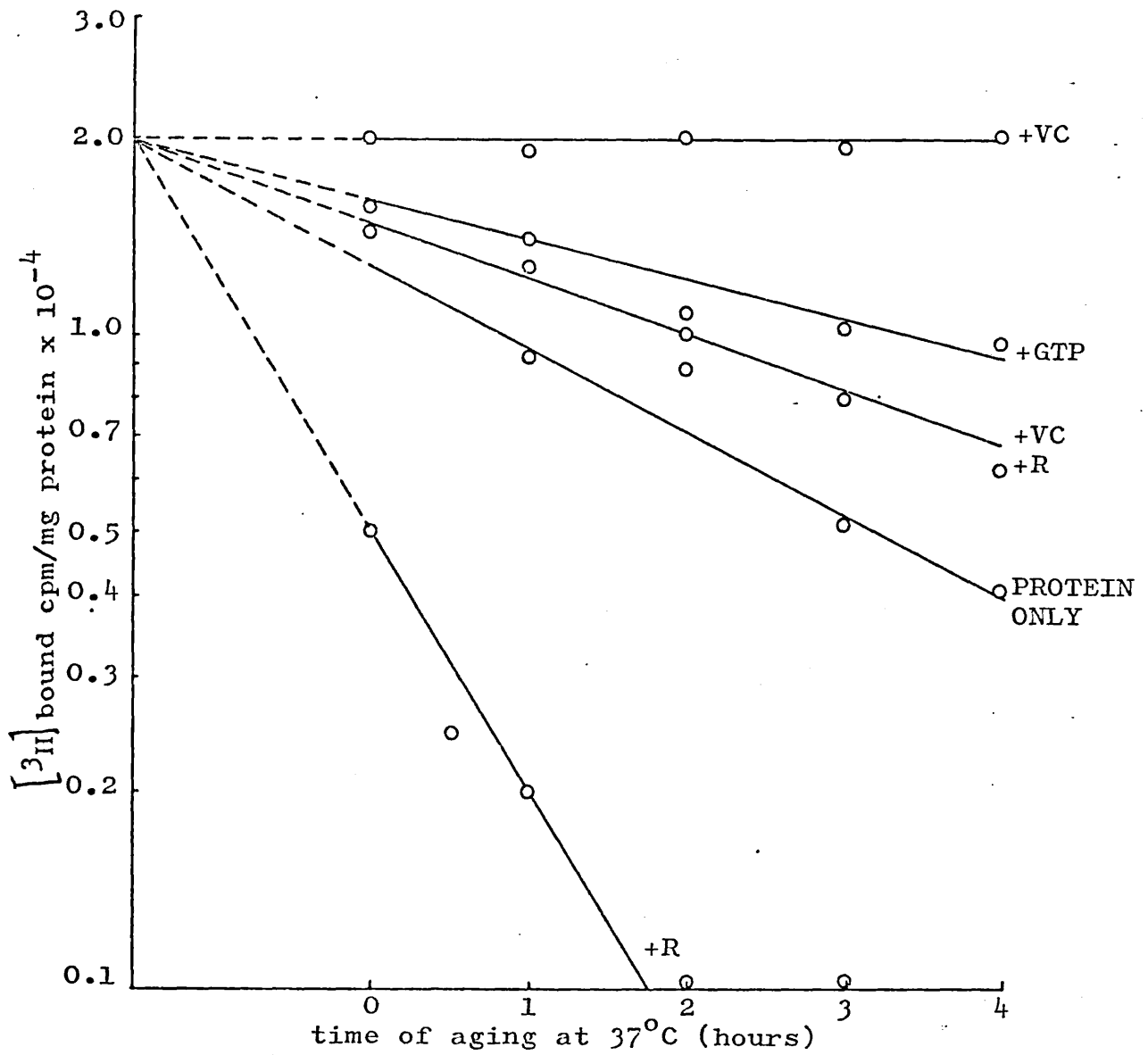
activity, it is tentatively concluded that a methoxy group on the indole is essential to obtain an effect on GTP binding.

#### 5.4 Effects of drugs on the aging of colchicine-binding activity

Wilson (1970) has shown that vinblastine stabilises the colchicine-binding activity of tubulin. A stimulatory effect on colchicine-binding activity can, therefore, be due to stabilisation of the protein-colchicine complex rather than to an effect on the binding reaction. This was initially investigated by measuring the decay of colchicine-binding activity on a partially purified tubulin preparation (second ammonium sulphate precipitate (3P) of batchwise procedure) in the presence of added vincristine ( $100 \mu\text{M}$ ). As shown in Fig. 5.3, vincristine increased the colchicine-binding activity of the preparation by about 100 %, as expected, before aging at  $4^{\circ}\text{C}$ , and this effect was maintained in preparations which had been aged up to 7 h at  $4^{\circ}\text{C}$ . However, after 24 h at  $4^{\circ}\text{C}$ , virtually all the colchicine-binding activity was lost in the presence or absence of vincristine. It is notable, nevertheless, that the initial rates of decay of colchicine-binding activity were nearly the same in the presence or absence of vincristine.

Similar experiments were carried out on the aging of the colchicine-binding activity of purified tubulin at  $37^{\circ}\text{C}$  in the presence of drugs ( $100 \mu\text{M}$ ) or GTP ( $100 \mu\text{M}$ ). These data, when plotted on a semi-logarithmic scale, as shown in Fig. 5.4, reveal that vincristine and GTP prevent or retard the first-order decay of colchicine-binding activity. In contrast, reserpine increased

Fig.5.4. Effects of various compounds on the aging of colchicine-binding activity of chromatographically purified foetal rabbit brain tubulin



Vc (vincristine), R (reserpine) and GTP were added at final concentration of  $100 \mu\text{M}$  where indicated. Experimental details as described in text, section 5.4.

the rate of decay. In this particular experiment (see Fig. 5.4) the half-life of colchicine-binding activity of purified tubulin, in the absence of added drug, was found to be 150 min. In the presence of vincristine or GTP, the half-life was extended to 330 min and 270 min, respectively. On the other hand, reserpine at the same concentration reduced the half-life to approximately 50 min. Attempts to reverse the reserpine effect by the simultaneous addition of equimolar concentrations of vincristine resulted in an increase of the half-life to an intermediate level of 210 min. By extrapolating each line back to include the incubation period for the colchicine-binding reaction (i.e. 1.5 h at 37°C), it can be seen that none of the additions had any effect on the initial colchicine-binding capacity of tubulin (Fig. 5.4).

From these data, it is obvious that Vinca alkaloids and GTP increase colchicine-binding activity by stabilising the tubulin molecule against thermal decay, while reserpine decreased the apparent binding activity by increasing the rate of decay of colchicine-binding sites. The fact that these two effects are antagonistic might indicate that the two drugs are acting on a common site.

#### 5.5. Effects of reserpine on rat brain after in vivo administration

Data from the study on the effects of indole alkaloids and structurally related compounds on the binding properties of tubulin, suggested a further investigation of their effects on the colchicine-binding activity and subcellular distribution of



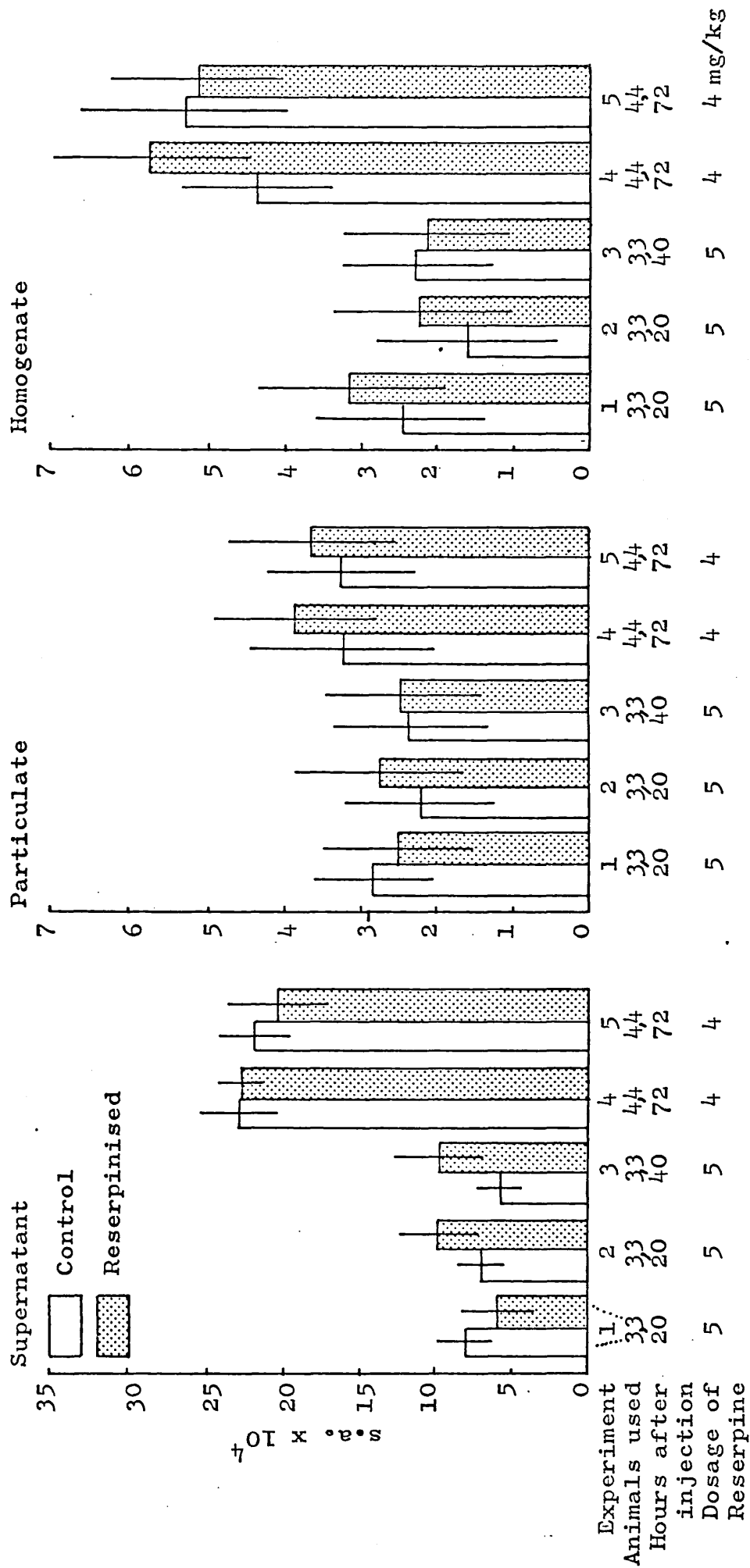
microtubular protein after administration of the drugs in vivo. Reserpine was chosen for this study in view of its inhibitory effect on the binding of colchicine and its marked activating effect on the binding of GTP to purified tubulin.

5.5.1 Effects of in vivo administration of reserpine on colchicine-binding activity in soluble and particulate fractions of rat brain

In this series of experiments, 3-4 female hooded rats were injected intraperitoneally with reserpine at concentrations of 2 - 5 mg/kg body weight and the same number of control litter mates were injected with the same volume of propylene glycol-propanol carrier solution described in 5.2.1. It was noticed that reserpinised animals were sedated for up to 12 h after injection, after which they showed complete recovery. The animals which were injected with carrier solution behaved normally. The animals were given free access to food and water and were left for 20 or 72 h before they were killed by decapitation after light ether anaesthesia.

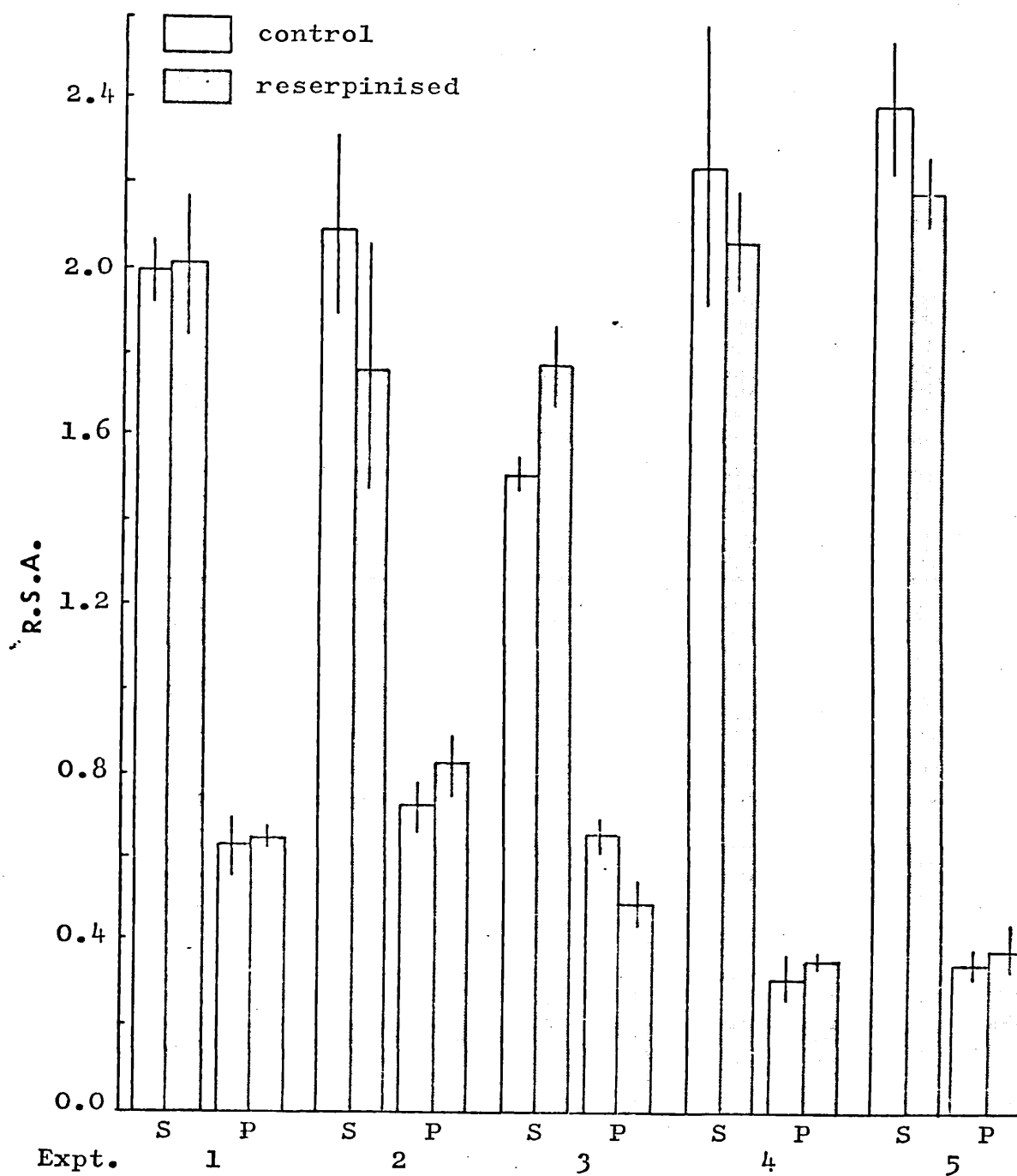
One cerebral hemisphere from each animal was homogenised in four volumes of P-Mg-GTP buffer, pH 6.8 containing 0.24 M-sucrose, and an aliquot of this homogenate was centrifuged at 4°C for 1 h at 100,000 g. The resulting pellet was resuspended in the same volume of homogenising medium. The homogenates, supernatants and particulate fractions were assayed for colchicine-binding activity by the DE 81 filter disc method. The protein concentration of each fraction was

Fig.5.5. Subcellular distribution of colchicine-binding activity of rat homogenates from control and reserpinised animals



Brains obtained from control animals which had been injected with reserpine carrier solution and from animals which had been injected with reserpine with the stated dosage and after the indicated intervals, were centrifuged at 100,000g x 60 min to obtain supernatant, particulate and homogenate fractions. The colchicine-binding activity of these fractions were assayed (see Chapter II 2.3.4.).

Fig.5.6. Relative distribution of the colchicine-binding activity in soluble and particulate extracts of brains from reserpinised and control rats.



$$\text{R.S.A.} = \frac{\% \text{ of bound cpm recovered}}{\% \text{ of protein recovered}}$$

Vertical bars indicate standard deviation (S.D.).

determined by the method of Lowry et al. (1951). The results were expressed in terms of specific activity (S.A. : bound cpm/mg protein) and relative specific activity (R.S.A. : % recovered cpm/ % recovered protein). In each experiment the means, with standard deviation, of the specific activities for the three fractions (i.e. homogenate, supernatant, and particulate) obtained from reserpinised animals were compared with the values for control animals.

As shown in Fig. 5.5 & 5.6, it is apparent that there are no statistically significant differences between the S.A. or R.S.A. values for colchicine-binding activities of the three fractions assayed between the two experimental groups. Experiment 3 is the only exception in which the R.S.A. of the supernatant fraction of reserpinised animals was significantly higher than that of the control animals, while the R.S.A. for the particulate fractions was significantly lower than that for the control animals (see Fig. 5.6). These data indicate that reserpine when injected in vivo at a concentration from 2 - 5 mg/kg body weight, has no significant effect on colchicine-binding activity in the soluble and particulate fractions of brain extracts.

After intraperitoneal injection, it has been estimated that only a very small fraction (1 %) of reserpine reaches the brain and besides, it is constantly being metabolised in vivo (Maggiolo & Haley, 1964). It is therefore, possible that the lack of effect of reserpine on colchicine-binding activity seen in vivo experiments may be due to the fact that the concentrations of reserpine present in brain tissue after injection would be far below those which had been found necessary to produce inhibition of colchicine-binding activity in vitro (cf. section 5.2.3).

5.5.2 The uptake and binding of colchicine in cerebral cortex slices from reserpinised rat

Tissue slice experiments were performed in collaboration with M. Reddington on cerebral tissue obtained from the same reserpinised and control animals as were used in the experiments described above.

One cerebral hemisphere from each reserpinised or control animal was chopped into slices of 0.33 mm thick using the McIlwain Tissue Chopper (see McIlwain & Rodnight, 1962) and portions of approximately 100 mg tissue were incubated for 5 min or 40 min in 3 ml of oxygenated 10 mM-tris-HCl buffer, pH 7.4 containing 128 mM-NaCl, 6.3 mM-KCl, 2.8 mM-CaCl<sub>2</sub>, 1.3 mM-MgCl<sub>2</sub>, 10 mM-glucose and [<sup>3</sup>H]-colchicine (2.5 μM; specific activity 4.45 x 10<sup>5</sup> cpm/nmole). After the incubation the tissues were rinsed and homogenised in 10 mM-tris-HCl buffer, pH 7.4 and centrifuged for 1 h at 100,000 g. Aliquots of each fraction (i.e. homogenate, supernatant and particulate fractions) were taken for determination of bound colchicine by the filter disc assay. A sample of supernatant was directly counted for radioactivity and taken as 'total uptake of colchicine'. The same procedure was carried out on control animals injected with the same volume of reserpine carrier.

In animals reserpinised for 20 h, the total uptake of colchicine into brain slices was increased by 20 % as compared to controls, after incubation for 5 min with [<sup>3</sup>H]colchicine (see Table 5.3). Longer incubations (40 min) resulted in a three-fold increase in the amount of colchicine bound, but under these conditions, no difference in uptake was seen as between tissue taken from reserpinised or control animals. The total amount of colchicine bound to brain tissue had also increased by 83 % in

Table 5.3

The effect of reserpine on the uptake and distribution of  ${}^3\text{H}$  Colchicine in guinea pig cerebral cortex slices.

Measurements	Time of Incubation (min)	20 hours		72 hours		% Change Reserpinised (pmoles colchicine/mg protein)	% Change
		Control (pmoles colchicine/ mg protein)	Reserpinised (pmoles colchicine/ mg protein)	Control (pmoles colchicine/ mg protein)	Reserpinised (pmoles colchicine/ mg protein)		
Total Colchicine Uptake	5	76±1	91±9	+19	78±5	73±2	-7
	40	79±6	78±12	-1	110±15	89±10	-19
Total Colchicine bound	5	8.5±1.4	15.6±3.6	+83	12.3±2.3	12.4±2.3	0
	40	27.0±3	28 ±4	+4	35.0±6	31.0±5	-12
Colchicine bound to supernatant	5	3.7±0.8	6.5±1.4	+75	4.8±1.8	5.3±1.1	+10
	40	11.4±1.7	12.1±3.9	+6	12.4±2.7	11.6±2.5	-6
Colchicine bound to pellet	5	4.8±0.7	9.1±2.4	+89	7.5±0.8	7.2±0.8	-4
	40	15.7±1.9	16.6±0.7	+6	23.1±4.4	19.5±4.5	-16

Values given represent the means of triplicate determinations ± S.D. from a single experiment. Control = tissue taken from animals injected with reserpine carrier solution ; reserpinised = tissue taken from animals injected with 4 mg/ body weight of reserpine and killed at times indicated. For further details see text, section 5.5.2.

reserpinised animals as compared to the control animals after 5 min incubation with colchicine. However, only 4 % increase was detected in samples incubated for 40 min. Similarly, after incubation for 5 min with colchicine, the amounts of colchicine bound to the supernatant and particulate fractions were correspondingly higher for tissue from reserpinised animals than from that taken from control animals. Here again, no differences in binding were found when incubations were carried out for 40 min.

When the same experiment was carried out on animals, 72 h after injection of reserpine, very little difference was found between control and reserpinised animals in either the total colchicine uptake, or in the amount of bound colchicine uptake, or in the amount of bound colchicine in the supernatant and particulate fractions, whether the tissue was incubated for 5 or 40 min (see Table 5.3).

The increase in the binding of colchicine in both supernatant and particulate fractions which was found 20 h after drug injection and after 5 min incubation with colchicine may reflect to some extent the increase in total uptake of colchicine seen in reserpinised animals. Moreover, it can be seen that the ratio of colchicine bound in the supernatant and pellet fractions of control and reserpinised tissue remains approximately the same. It is possible that reserpine might act directly or indirectly, by affecting the permeability of cerebral cortex slices to colchicine under the condition specified (see also Giochetti & Shore, 1970). Further experiments to study in detail the kinetics of colchicine uptake and binding in brain slices are necessary before these results can be satisfactorily discussed. One possible

approach which remains to be explored would be to test~~ed~~ the effects of reserpine more directly during incubation of tissue slices in the presence of the drug.

## 5.6 Discussion

At first, it was thought that the inhibitory effect of reserpine on colchicine-binding activity of tubulin might be due to the trimethoxybenzoic acid moiety which also forms part of the colchicine molecule. When trimethoxybenzoic acid and a non-indole compound, tetrabenazine (Roche) which competes for reserpin-binding sites (Quinn et al., 1959), were tested on both binding activities of tubulin, they were found to have little effect. It became apparent that the effects of reserpine on the binding activities of tubulin might be due to its indole moiety. Thus, other indole compounds tested such as melatonin, harmine, yohimbine, 5-methoxyindole and serotonin all affected one or both of the binding activities, although some indole compounds such as 6-methoxyindole and tryptamine had no apparent effect. It is noteworthy that the methoxy indoles tested consistently increased the GTP-binding activity of tubulin. The finding that harmine only increased GTP-binding activity at high concentration (500  $\mu$ M) might indicate that this compound acts through some secondary steric effect. On the other hand, methoxy indoles have opposite effects to Vinca alkaloids on the colchicine-binding activity of tubulin, and this antagonistic effect cannot be simply explained by their difference in chemical structures. However, an explanation for this discrepancy can be drawn from the work of Banerjee et al. (1972) and Margulis (1973) on the Stentor



ciliated oral membranellar band regenerating system which consists of microtubular structures very sensitive to mitotic spindle inhibitors. The delay in regeneration of the oral membranellar band, i.e. of reassembly of microtubules, which was seen in the presence of antimitotic drugs such as colcemid and popdphyllotoxin, has been used by these authors as an assay to determine the potency of antimitotic drugs. In this system, they found that indole compounds such as melatonin and serotonin delayed the regeneration of Stentor membranellar band. As a result of this work, Margulis (1973) postulated that in a compound "at least one methoxy substitution on an aromatic ring approximately  $7\text{\AA}$  away from an electronegative atom bound to hydrogen (-NH or -OH group) is required for the generation of a reproducible, exponential delay curve" in the Stentor membranellar regeneration system. Since these compounds act on microtubule assembly, it was postulated that these structural requirements reflect those of microtubular subunit protein. In the present work, the indole alkaloids and indole amines which affect the colchicine- and GTP- binding activities of tubulin, to some extent fulfil the requirements stated in her working hypothesis.

It is also conceivable that drugs which increase colchicine-binding activity, may also induce the depolymerisation of microtubules into tubulin dimers, while drugs which inhibit colchicine-binding activity may do so by causing tubulin dimers to form polymers or microtubular structures. There is now reasonable evidence that colchicine does not bind to or disrupt polymerised (i.e. microtubular) forms of tubulin in vitro (Olmsted & Borisy, 1973). Since GTP is required for the polymerisation of tubulin into microtubules (Shelanski et al.,

1973), any drug which induces the assembly of microtubular structures could also act by increasing and / or stabilising the binding of GTP to the protein. This working hypothesis was tested by examining the effects of drugs on the assembly of microtubules in vitro, and the results obtained are described in the next chapter.

Chapter VI      The effects of drugs on the assembly of microtubule  
in vitro

6.1            Introduction

6.2            Methods

6.2.1          Preparation of microtubules

6.2.2          Preparation of drugs

6.2.3          Addition of drugs

6.2.4          Electron microscopy

6.2.5          Polyacrylamide gel electrophoresis

6.3            Results

6.3.1          Preliminary studies on the polymerisation of microtubules

6.3.2          Effects of drugs on the polymerisation of microtubules

in vitro

6.3.3          Effects of drugs on polymerised microtubules

6.4            Discussion

Chapter VI      The effects of drugs on the assembly of microtubule  
in vitro

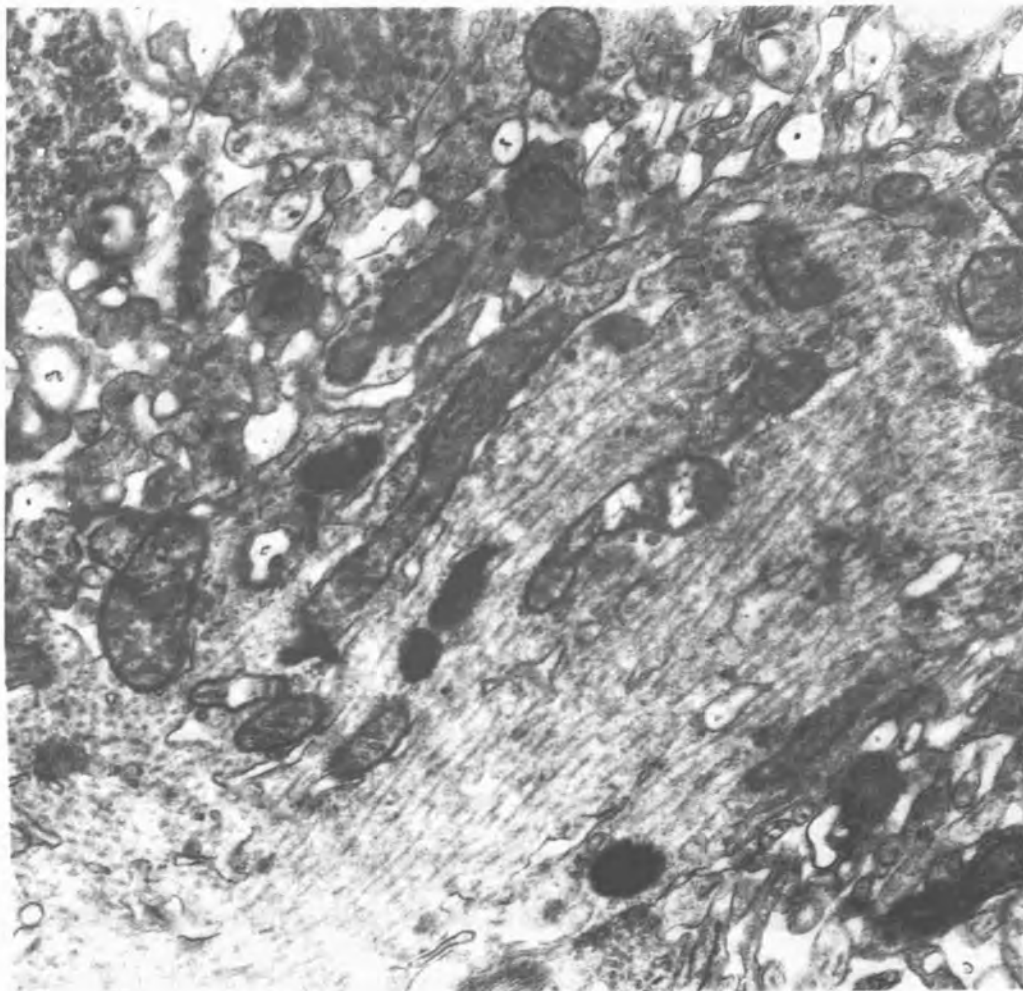
6.1      Introduction

Antimitotic drugs such as colchicine (Eigsti & Dustin, 1955) act by disrupting microtubules; further, in vivo administration of colchicine or the antimitotic Vinca alkaloids leads to the disappearance of microtubules from nerve cells, (for normal microtubules see Fig.6.1) with the formation of 100 Å diameter filaments (Wisniewski et al., 1968) and paracrystalline inclusions (Bensch & Malawista, 1969; Marantz & Shelanski, 1970). Whether these observed morphological changes are due to a direct effect of these drugs on the microtubular system, or arise secondarily as a result of the interactions of these drugs with other cellular sites is still uncertain. Until recently, it has not been possible to study the direct effect of these drugs on microtubular systems. However, the demonstration that microtubules can be reassembled from tubulin subunits in vitro (Weisenberg, 1972; Shelanski et al., 1973) now provides an opportunity to test the effect of these drugs on the assembly and stability of microtubules in vitro.

In this chapter, the formation of microtubules by polymerisation in vitro from their protein subunits has been employed as a model for studying the effects of various drugs on both the process of polymerisation, and on the appearance of the reassembled microtubular structures.

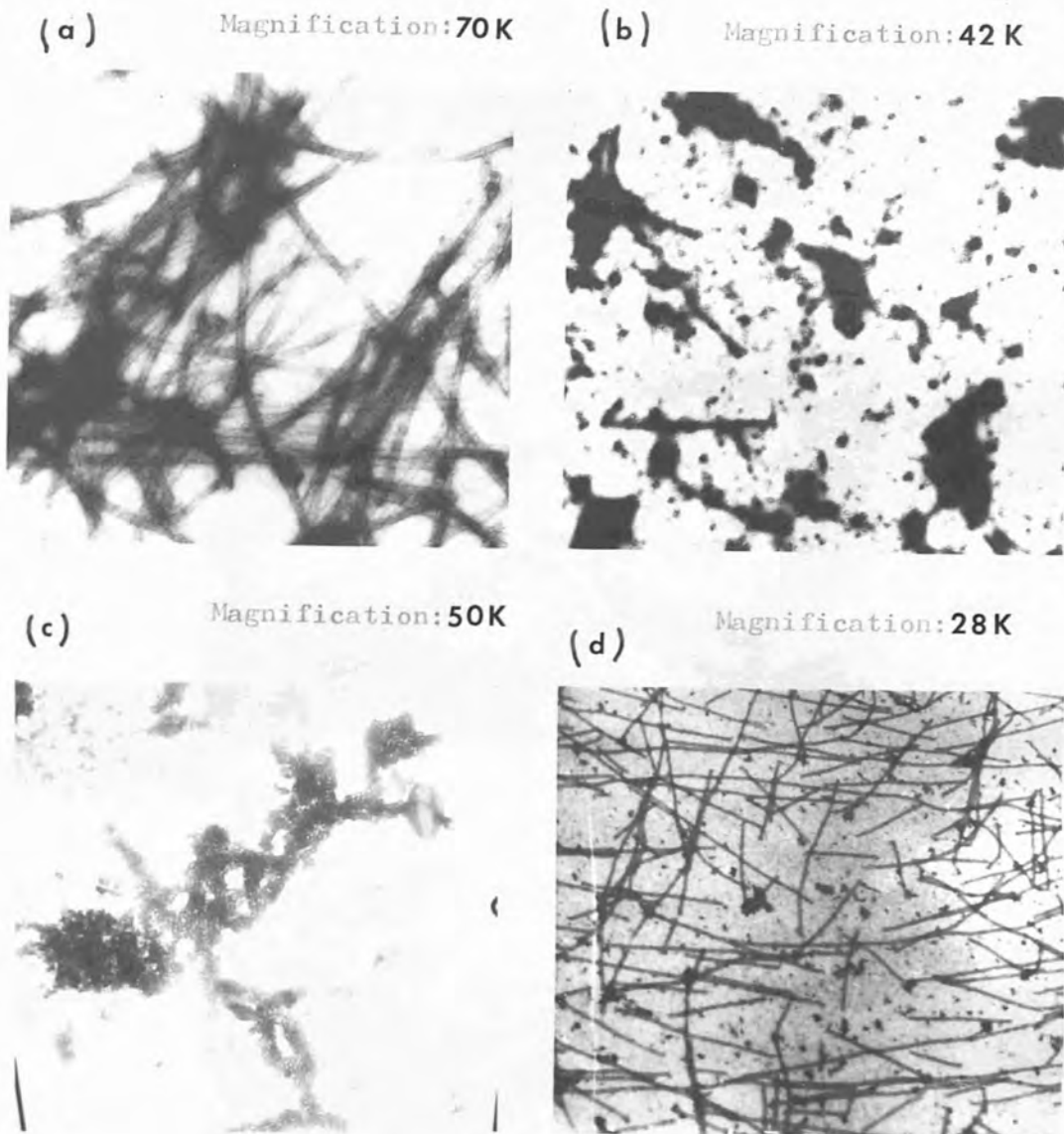
**Fig.6.1.** Microtubules in guinea pig brain cortex fixed and stained in situ

---



Magnification : 10 K

Tissue was fixed in glutaraldehyde and stained with lead citrate.



- (a) Control
- (b) Colchicine ( $40\mu\text{M}$ )
- (c) Vincristine ( $125\mu\text{M}$ )
- (d) Reserpine ( $100\mu\text{M}$ )

### 6.2.2 Preparation of drugs

Drugs were prepared as solutions having a concentration 10 times higher than that required in the final reaction mixture. For an experiment, 1 volume of the drug solution was added to 9 volumes of solution containing microtubular protein. The following drugs were dissolved in double glass distilled water: colchicine, vincristine, vinblastine, leurosine and leurosidine. Melatonin was dissolved in 4 % (v/v) ethanol in water, and reserpine in a propylene glycol-propanol carrier solution as in Chapter V, 5.2.1. All control incubations (i.e. minus drugs) were carried out in the presence of an equivalent volume of the appropriate drug carrier solution.

### 6.2.3 Addition of drugs

To test the effects of drugs on the polymerisation process, an appropriate volume of the drug was added to a sample of freshly prepared supernatant fraction (S3) (containing free microtubular subunits; see Chapter II, 2.2.3) and the mixture was preincubated for 10 min at 37°C before the addition of an equal volume of assembly buffer; incubation was then continued for 20 min (i.e. total time of incubation at 37°C, 30 min).

To examine the effects of drugs on the appearance of reassembled microtubules, drugs were added to the supernatant fraction (S3) which had been previously incubated for 20 min at 37°C with an equal volume of assembly buffer. This solution, containing reassembled microtubules, was then incubated for a further 10 min at 37°C in the presence of the drug.

After incubation under the appropriate condition samples were taken for electron microscopy either directly, or from the pellet (P4), obtained by centrifugation (at room temperature) for 1 h at 100,000 g, which was resuspended in a small volume of MES buffer. In the latter case, observation of microtubules under the electron microscope was facilitated due to their higher concentration.

#### 6.2.4 Electron microscopy

A drop of sample was mounted onto a G400 Formvar carbon coated grid held with a pair of fine forceps. One drop of 1 % aqueous uranyl acetate was added and the mixture was quickly drained off from the grid by touching the side with absorbant. The grid was then ready for viewing under the electron microscope. Two grids were normally prepared for each sample. Grids containing negatively stained samples were viewed in an AEI 6B or an AEI Corinth 275 electron microscope maintained at 60 KW. Photographs were taken at low magnification (5,000 to 25,000x). Selected representative areas of the grids were also photographed at high magnifications (30,00 to 80,000x).

#### 6.2.5. Polyacrylamide gel electrophoresis

The purity of each preparation of microtubular protein was also assessed by polyacrylamide gel electrophoresis in the presence of SDS as described in Chapter II, 2.5.



### 6.3 Results

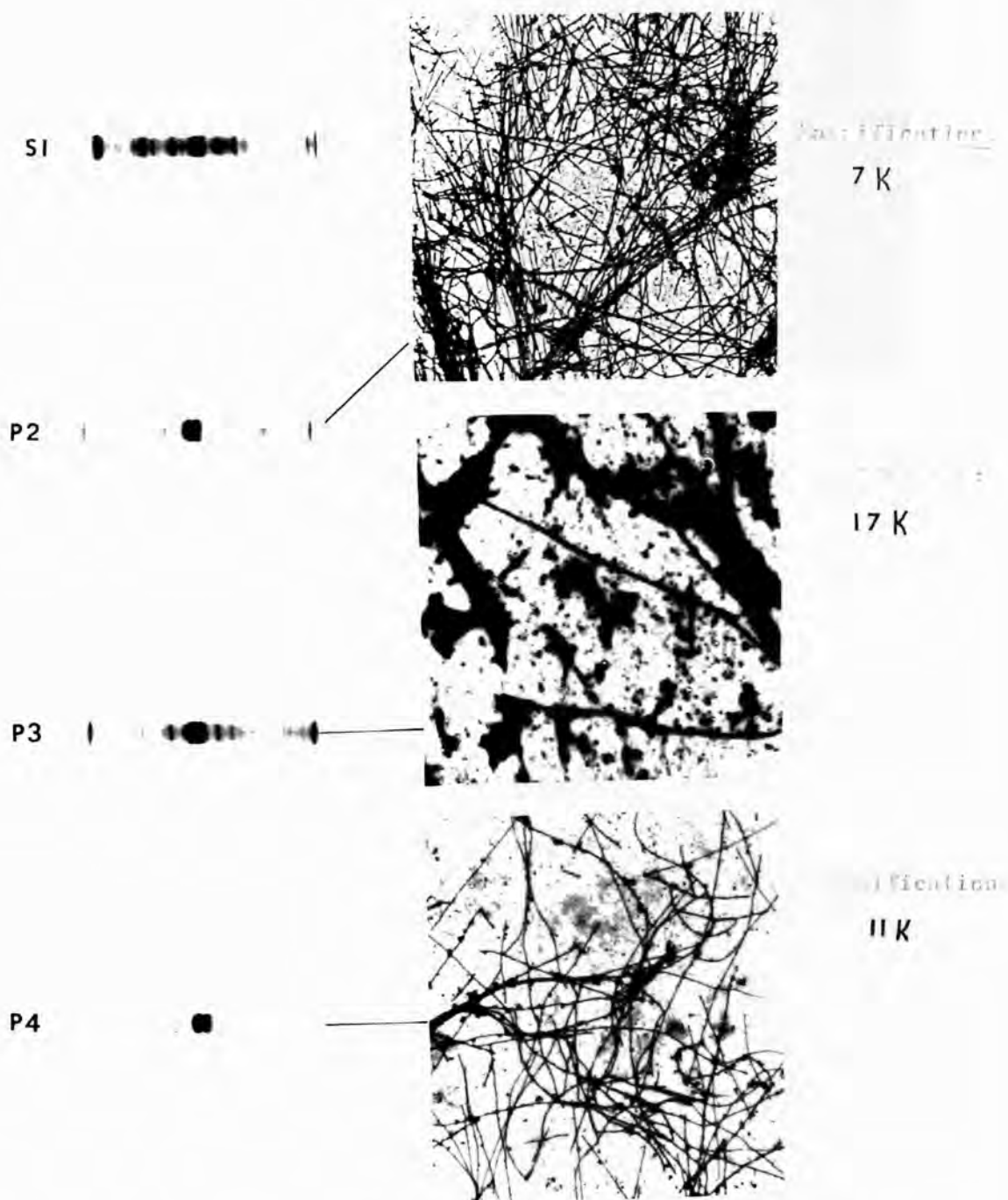
#### 6.3.1 Preliminary studies on the polymerisation of microtubules

Electron microscopic analysis, polyacrylamide gel electrophoresis and assays of colchicine-binding activity of samples taken at various stages of the preparation procedure showed that two cycles of polymerisation were sufficient to obtain relatively pure microtubules (see Fig. 6.2).

When examined under the electron microscope, the sample from the pellet (P2) obtained by centrifugation after the first polymerisation, showed an abundance of microtubules with some non-tubular contaminants (Fig 6.2). The non-polymerisable material was removed as pellet (P3) after centrifugation in the cold of the disassembled microtubules, and this pellet consisted mainly of tubular aggregates in addition to intact microtubules as seen in Fig. 6.2. The second reassembly step yielded a more homogeneous sample of microtubules in the pellet (P4) obtained after high-speed centrifugation at room temperature (Fig. 6.2). Polyacrylamide gel electrophoresis of the above samples showed a progressive increase in purity of microtubular proteins in comparison to the starting brain soluble extract (S1), as shown in Fig. 6.2. The pellet (P2) obtained after the first polymerisation step consisted mainly of the  $\alpha$  and  $\beta$  subunits of tubulin, but in addition it contained two distinct high-molecular weight proteins which remained near the origin, and a number of other minor components present in trace amounts (see Fig. 6.2). Samples of the pellet fraction P3 (Fig. 6.2) showed a protein pattern similar to that found in the first pellet (P2), while the final pellet containing microtubules (P4) which was

Fig.6.2.

Electron micrographs of polyacetylene fibers prepared by electrochemical polymerization of acetylene in the presence of various amounts of dodecylamine.



Electrolytic cell for electrochemical polymerization of acetylene. The left-hand side of the cell is connected to the positive terminal of the power source and the right-hand side to the negative terminal. The cell contains a solution of acetylene and dodecylamine. The polymerization is carried out at a constant potential. The resulting polyacetylene fibers are collected on a cathode. The fibers are then washed and dried. The molecular weight distribution of the fibers is determined by gel permeation chromatography (GPC). The GPC chromatograms show the distribution of the fibers in terms of molecular weight. The electron micrographs show the morphology of the fibers. The fibers are generally composed of many individual polymer chains that are intertwined to form a network. The network structure is more dense for higher molecular weight fractions and more porous for lower molecular weight fractions.

obtained after the second cycle of assembly, showed almost exclusively the  $\alpha$  and  $\beta$  subunits of tubulin, and the two minor high molecular weight subunits near the origin (Fig. 6.2). The minor high molecular weight components were invariably present to about the same extent in all microtubular protein preparations derived from reassembled microtubules (see also Shelanski et al. 1973).

At each stage of the purification procedure the colchicine-binding activity was assayed by the DE 81 filter disc method (2.3.4) on cold-depolymerised microtubular protein samples. It was found that the colchicine-binding activity of fraction P2 and P4 was enriched by 7-fold and 9-fold, respectively, in respect to the binding activity measured in the initial fraction S1. Furthermore, the protein obtained by disassembly in the cold of the final microtubular pellet (P4) bound approximately  $0.5 \pm 0.2$  ( $n = 3$ ) moles of colchicine/mole of dimer (M.W. 110,000). This value agrees with those obtained for guinea pig brain microtubular protein prepared by the same method (see Reddington et al., 1975).

#### 6.3.2 Effects of drugs on the polymerisation of microtubules in vitro

As described in Chapter IV, reserpine inhibited the binding of colchicine to tubulin but activated that of GTP, while the Vinca alkaloids increased both the colchicine and GTP-binding activity of tubulin. The effects of melatonin were found to be very similar to those seen with reserpine (see Chapter V, 5.2 & 5.3) Colchicine, on the other hand, did not affect the binding of GTP to tubulin. The effects of these drugs on the formation and appearance of microtubules reassembled in vitro are

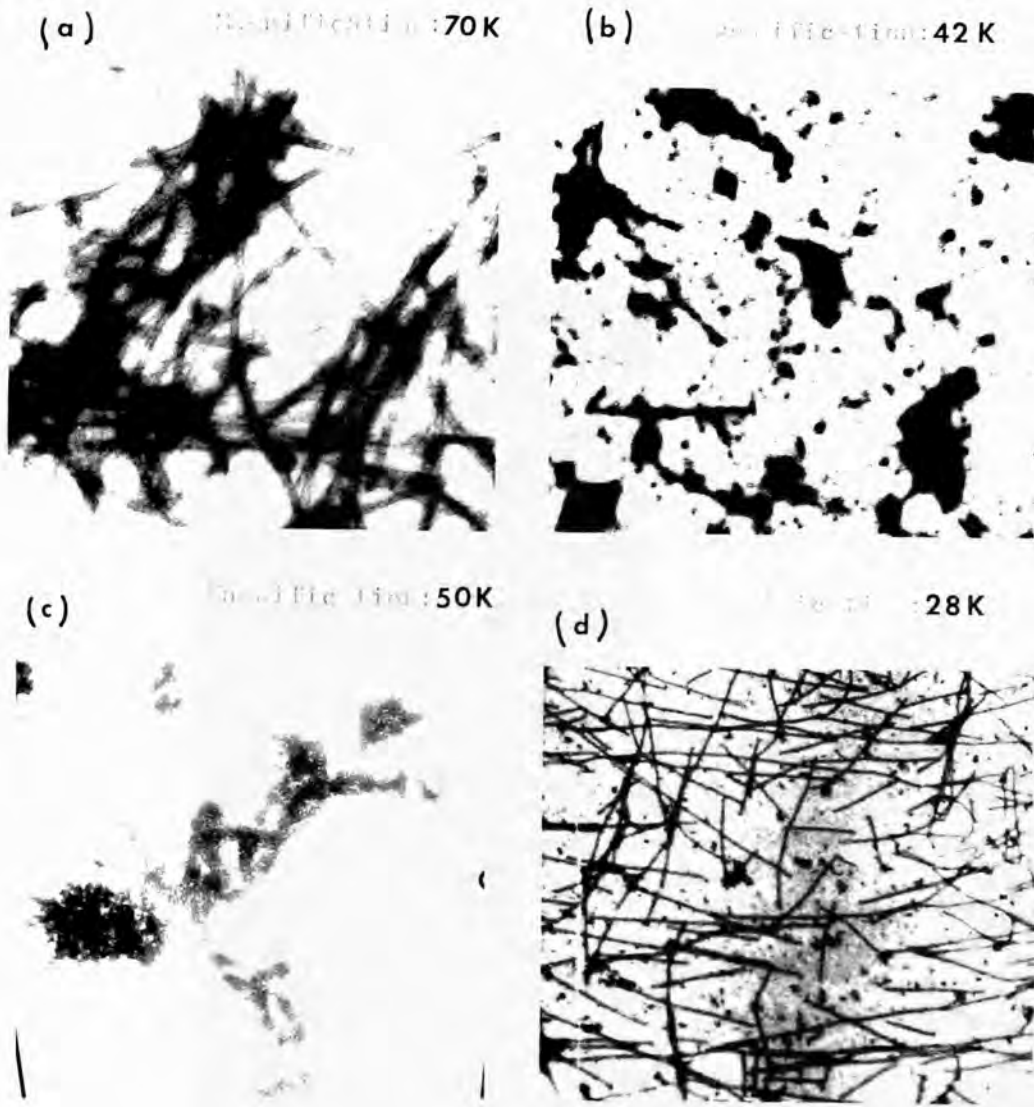
Fig. 6.3. to Fig. 6.7.

The effects of drugs on the in vitro reassembly  
of microtubules from foetal rabbit brain

---

Drugs were added at the concentration indicated  
before the last polymerisation step as described in  
the text, section 6.2.3.

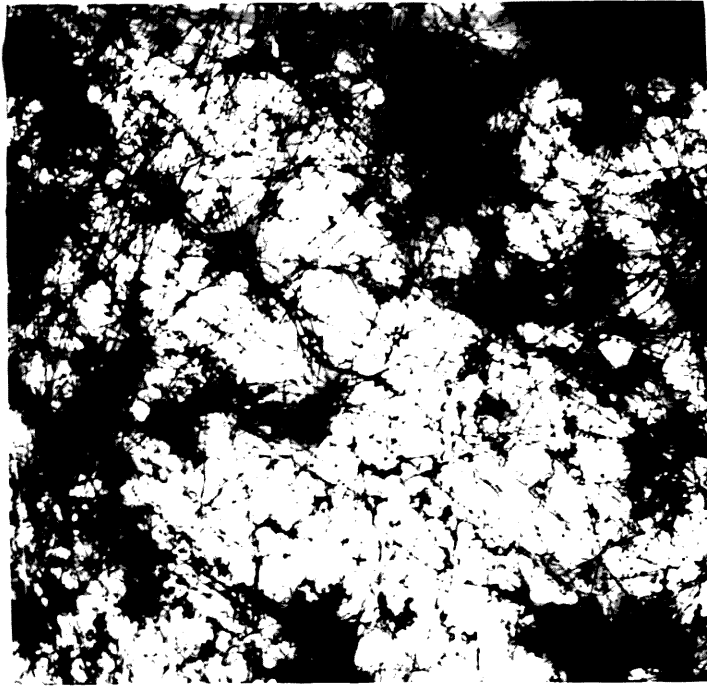
Samples were negatively stained with 1% aqueous  
uranyl acetate (see section 6.2.4.) for electron  
microscopy.



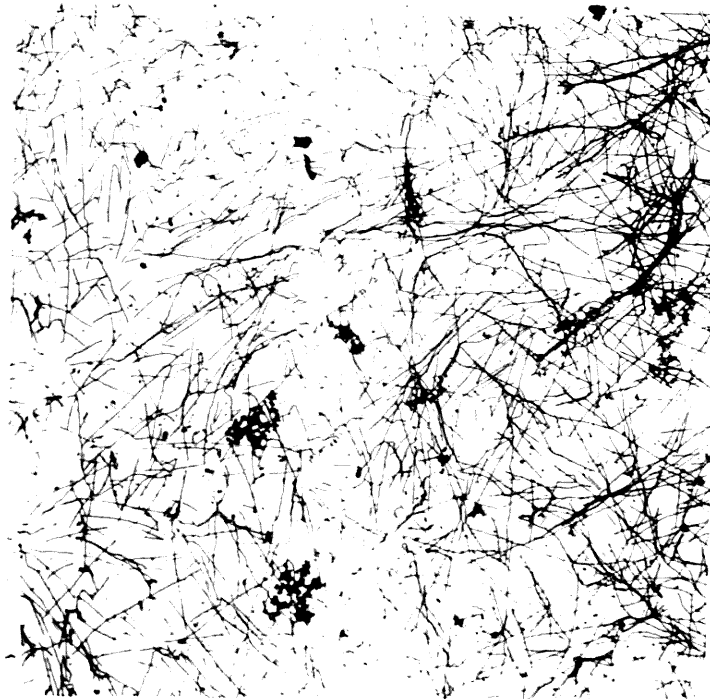
- (a) Control
- (b) Colchicine ( $40 \mu M$ )
- (c) Vincristine ( $125 \mu M$ )
- (d) Reserpine ( $100 \mu M$ )

Fig. 6.4.

(a) Magnification: 10K



(b) Magnification: 5K



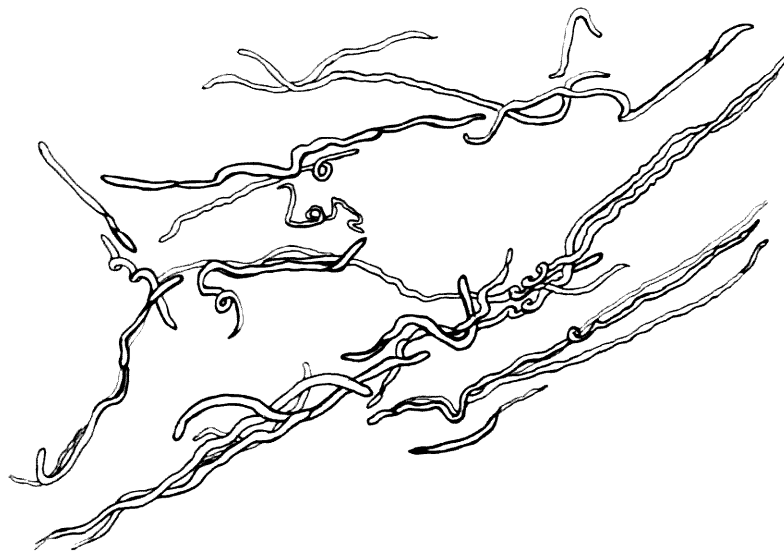
(a) Control (0.4% ethanol)

(b) Colatonic (1,000  $\mu$ M)

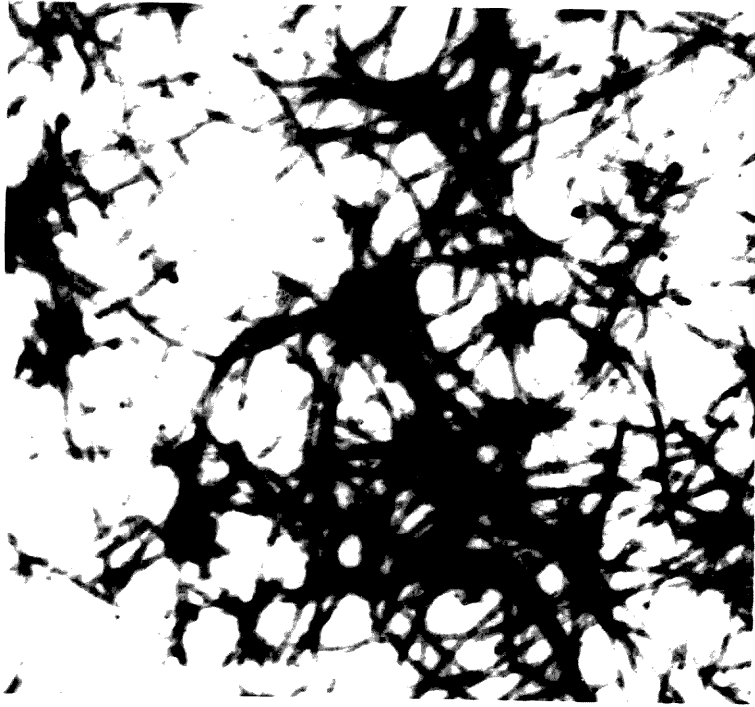
Helicobacter (1,000  $\mu$ m) Magnification: 28 K



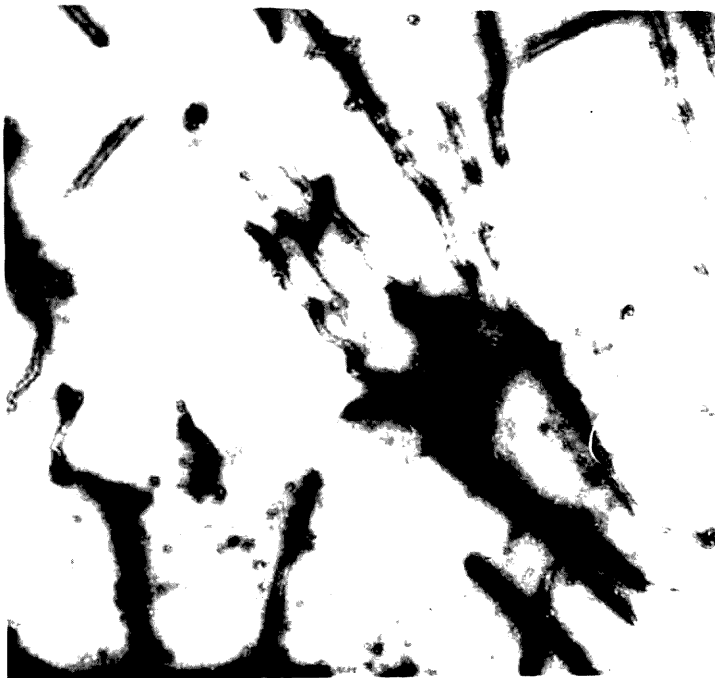
Diagrammatic illustration



(a) Magnification: 28K



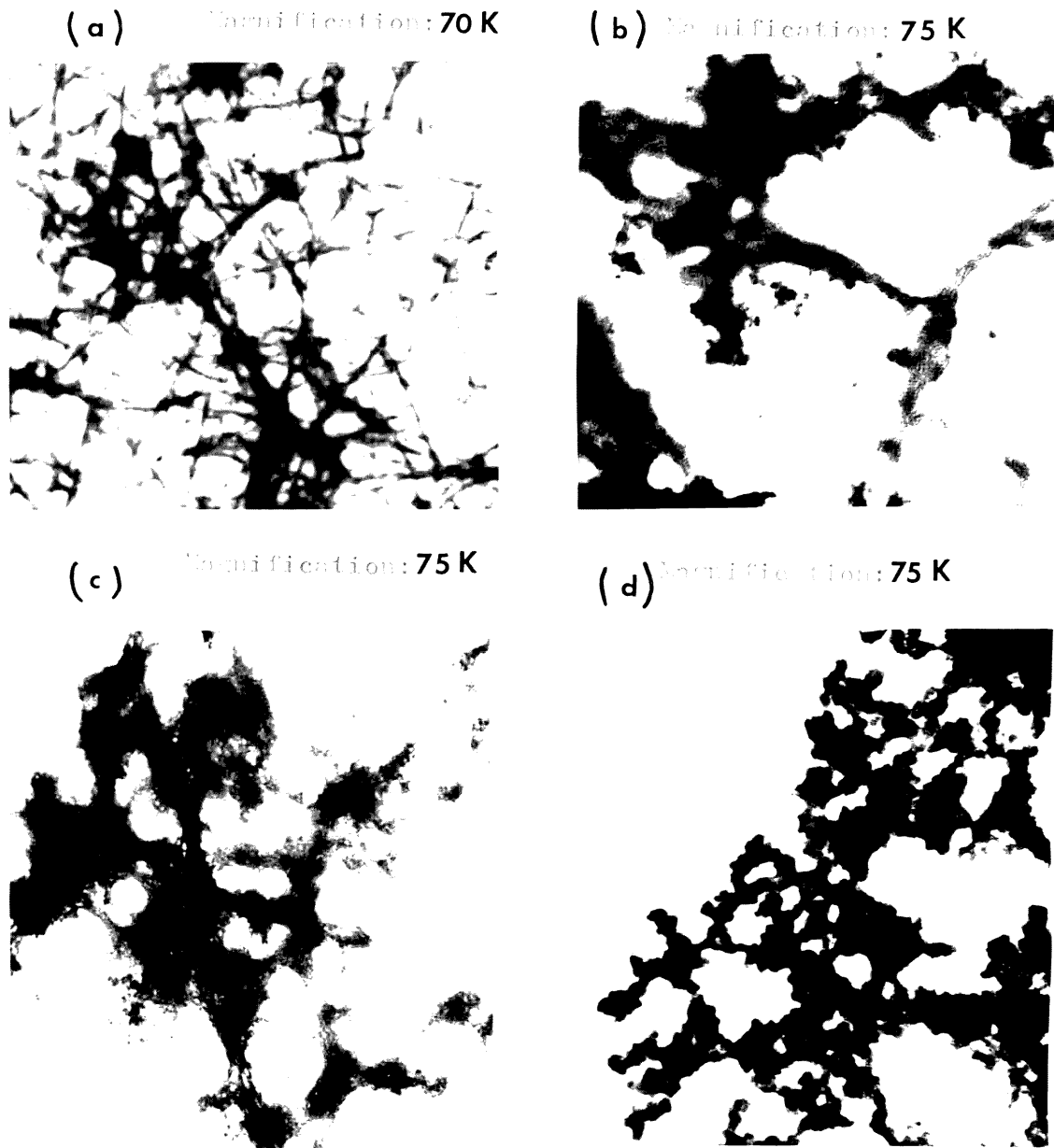
(b) Magnification: 40K



(a) Control (0.4% ethanol)

(b) Melatonin (1,000  $\mu$ M)





(a) Control  
(b) Vincristine ( $125 \mu M$ )  
(c) Leucosine ( $1,000 \mu M$ )  
(d) Leucosidine ( $1,000 \mu M$ )

described below.

In the first instance, tubulin subunits from polymerised microtubules were preincubated in the presence of the drugs and their ability to reassemble *in vitro* was studied.

At a concentration of 0.1 mM, reserpine had no obvious effect on the appearance of the polymerised microtubules. However, this drug appeared to promote tubule assembly and/or stabilise reassembled microtubules, and in general, the length of the tubules appeared to be greater than in control samples (i.e. samples incubated with the propylene glycol-propanol carrier alone) (Fig. 6.3d). Occasionally, some small aggregates were also observed in the reserpine-treated reassembled microtubules. However, the number and length of microtubules are not reliable criteria for quantitative analysis of electron micrographs under the conditions employed in the present study, since the reproducibility of sampling aliquots from pelleted material (P4) is doubtful.

Colchicine and vincristine sulphate had the most dramatic effects on the formation of microtubules. Colchicine, at a concentration of 40  $\mu$ M, completely prevented the formation of microtubules (Fig. 6.3b). A large number of globular aggregates of microtubular subunits was observed, with only very few short tubular elements. Vincristine sulphate, at a concentration of 0.125 mM, induced the formation of non-tubular aggregates (Fig. 6.3c): no microtubules could be detected under these conditions. In the absence of drugs, numerous normal microtubules were observed (Fig. 6.3a).

Melatonin, at a concentration of 1 mM, distorted the cylindrical appearance of microtubules, giving them a twisted

and 'curly' appearance (Fig. 6.4b). Some of them were observed to be intertwined with one another as shown in Fig. 6.5 & 6.6b. A control sample containing 0.4 % (v/v) ethanol (i.e. the carrier used to dissolve melatonin) exhibited normal microtubules with no sign of malformation (Fig. 6.4a & 6.6a). This phenomenon was observed in two separate experiments using different preparations of fresh microtubular protein. Lower concentrations of melatonin (0.5 mM and 0.1 mM) failed to produce such an effect in the same experiments.

Leurosidine and leurosine (1 mM) (for chemical formulae see Fig. 6.12) which are structural analogues of vincristine and vinblastine, prevented the normal formation of microtubules in vitro, but coiled-coils of protofilaments were observed (Fig. 6.7). No normal microtubules could be seen in the samples which had been treated with leurosidine and leurosine (Fig. 6.7c & d). No significant differences were observed between the effects of these two compounds on the formation of microtubules in vitro, or on their protein composition as analysed by polyacrylamide gel electrophoresis. In the appropriate control samples, normal microtubules were observed and no such coiled-coil structures occurred (Fig. 6.7a).

### 6.3.3 Effects of drugs on polymerised microtubules

Microtubules prepared by two cycles of assembly and disassembly were treated with drugs, in an attempt to distinguish between the effects of drugs on the polymerisation process, and a more direct effect on intact microtubules. The experimental procedure used is as described in section 6.2.3.

Melatonin (1 mM) and reserpine (0.1 mM) had no observable effect on the appearance of intact preformed microtubules

Fig. 6.8. to Fig. 6.10.

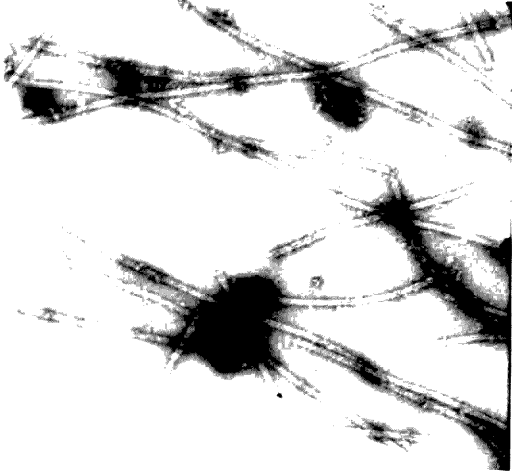
The effects of drugs on the stability of in vitro  
reassembled microtubules from foetal rabbit brain.

Drugs were added at the concentration indicated after polymerisation of microtubules as described in text, section 6.2.3.

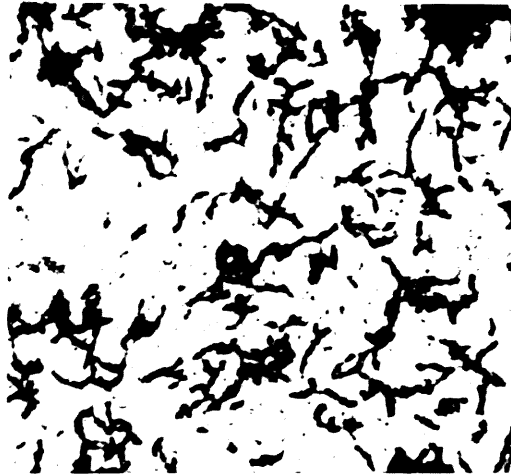
Samples were negatively stained with 1% aqueous uranyl acetate (see section 6.2.4.) for electron microscopy.

Fig. 6.8.

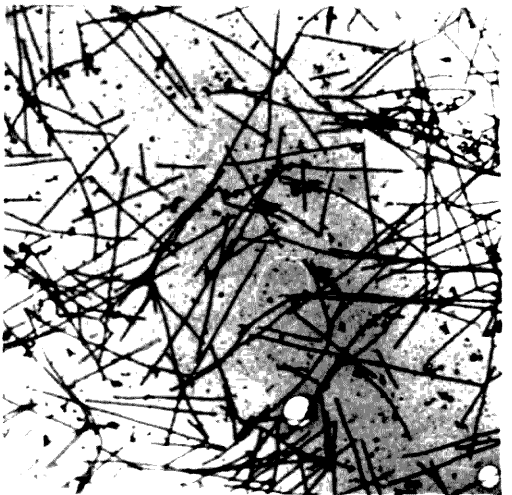
(a) Magnification: 67K



(b) Magnification: 10K



(c) Magnification: 5K



(d) Magnification: 15K



(a) Control

(b) Vincristine ( $125 \mu\text{M}$ )(c) Melatonin ( $1,000 \mu\text{M}$ )(d) Reserpine ( $100 \mu\text{M}$ )

Fig. 6.9

(a) Magnification: 60 K



(b) Magnification: 50 K

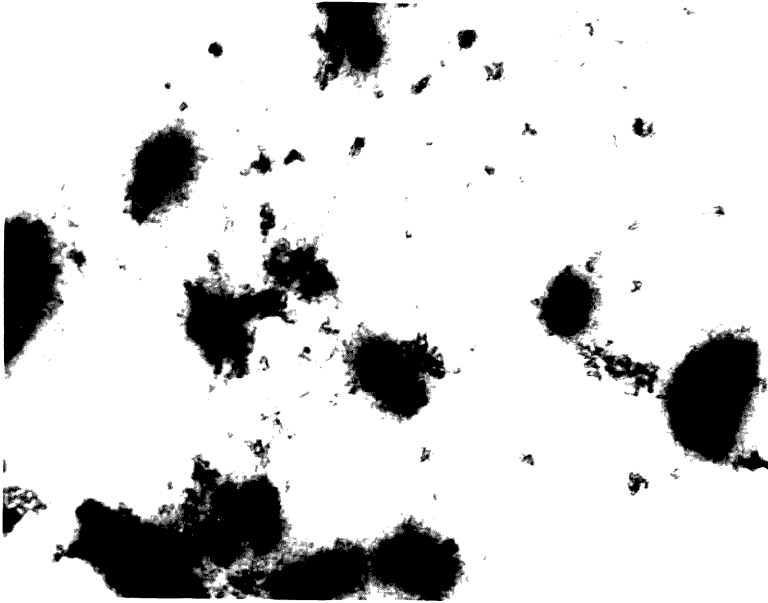


(a) Control

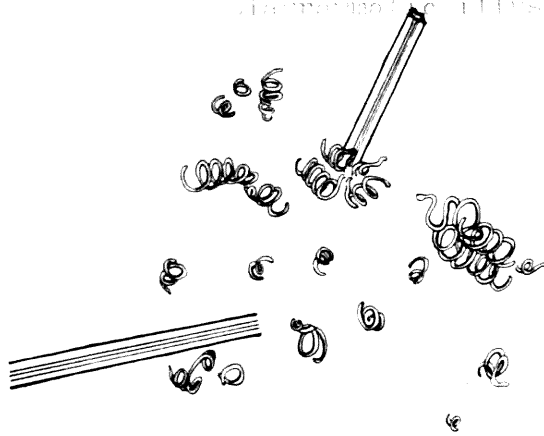
(b) Leucosine (100  $\mu$ M)

Fig. 6.10.

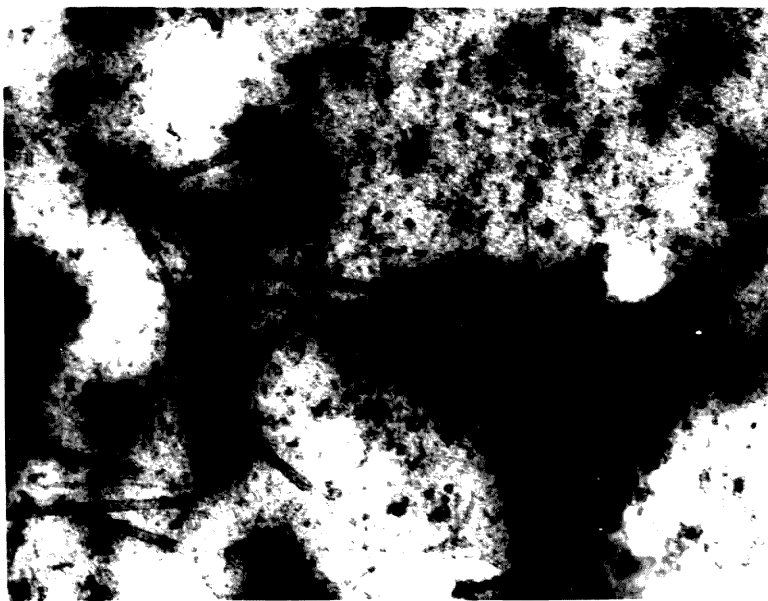
(a) Magnification: 100 k



Schematic illustration of (a)



(b)



Magnification: 100K

- (a) Ferric chloride (100  $\mu$ M)
- (b) Ferricyanide (100  $\mu$ M)

in vitro. Curly microtubules such as those obtained after treating tubulin subunits with melatonin before and during polymerisation (see above) were not observed (see Fig. 6.8c&d). Higher concentrations of these two drugs were not tested due to their limited solubility in the carrier solutions employed.

On the other hand, vincristine sulphate, at a concentration of 0.125 mM, invariably caused disruption of preformed microtubules giving rise to the appearance of non-tubular fibrous aggregates, as shown in Fig. 6.8b. These aggregates were similar to those seen when the same concentration of vincristine was added before the reassembly of microtubules (Fig. 6.3c). Leurosidine and leurosine sulphate, both at a concentration of 0.1 mM, behaved quite differently from vincristine since they seemed to disrupt the reassembled microtubules into their constituent protofilaments as shown in Fig. 6.9 & 6.10. The results obtained are consistent with the idea that leurosidine and leurosine disrupt the microtubules by acting at both ends, and causing them to fray (see Fig. 6.10). The microtubule is split into protofilaments which appear to coil up individually. The coiling up of protofilaments would then give rise to the spring-like or ladder-shape structures seen in Fig. 6.10. Single coils or ring-like structures were also observed and they probably represent short coiled up segments of protofilaments.



#### 6.4 Discussion

The in vitro formation of microtubules from their subunits provides a simple model system for studying the effects of drugs on the process of polymerisation and on the structural stability of intact microtubules. When added before the assembly of microtubules, colchicine prevents the formation of microtubules and under the same experimental conditions, vincristine leads to the formation of non-tubular aggregates. Higher magnification of this aggregate reveals the coiled-coil structures of protofilaments. The mechanism of these effects cannot be deduced from the data presented here, but clearly deserves further investigation.

Reserpine, though it has no obvious effect on the structure of intact microtubules, seems to promote the formation of apparently normal microtubules as judged by the electron micrographs. Therefore, it is possible that reserpine may exert an accelerating effect on the formation of microtubules, or alternatively it may stabilise the assembled microtubules. Since this accelerating or stabilising effect can hardly be detected by electron microscopic examination, it is suggested that viscometric (see Kuriyama & Sakai, 1974) or flow birefringence analysis (see Haga et al., 1974) of polymerisation in the presence of this drug might yield useful data.

The formation of distorted microtubules in the presence of melatonin could tentatively be explained in terms of an accelerating effect on the polymerisation process. When assembly of microtubules from their subunits takes place more rapidly than usual, erroneous arrangement of subunits could give rise to the formation of non-linear and curly microtubules.

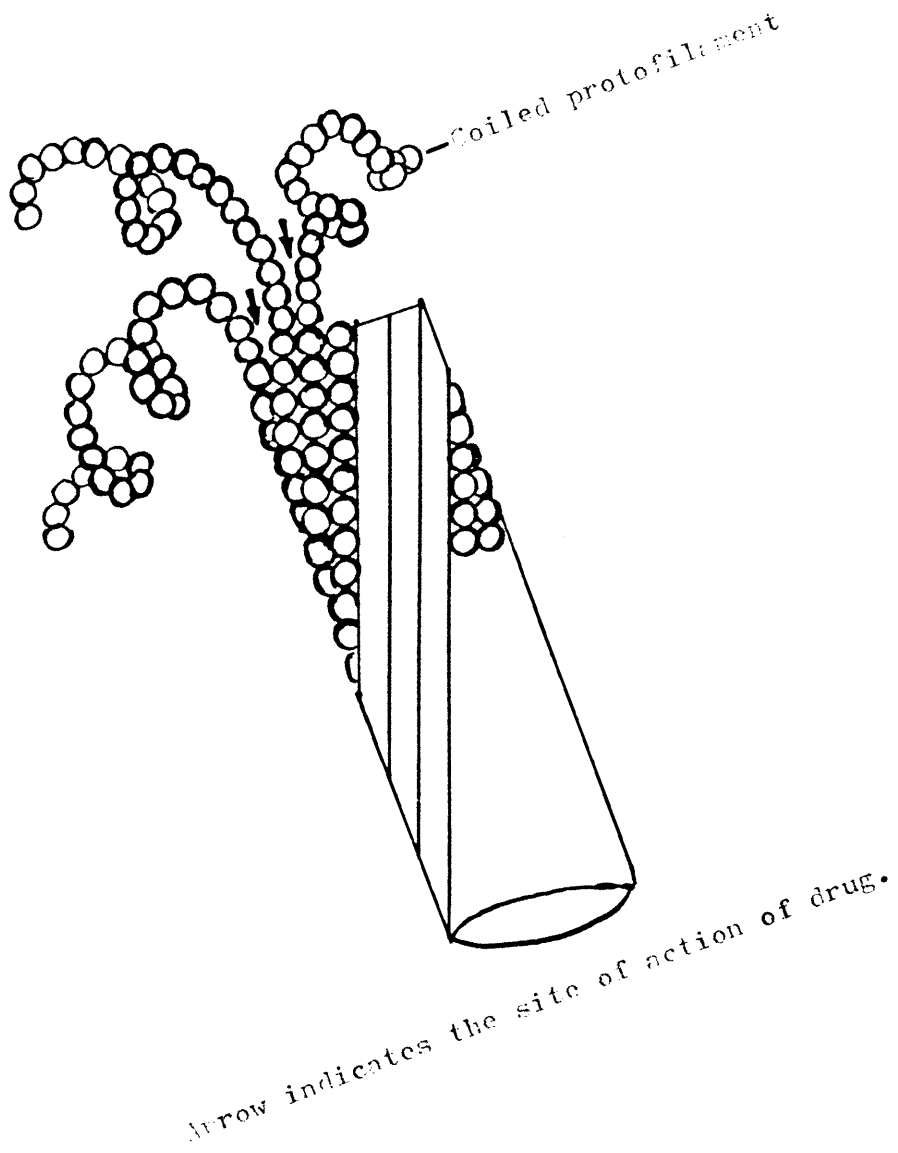
This interpretation will be further discussed in the last chapter in a wider context, in relation to its effects on the binding properties of tubulin.

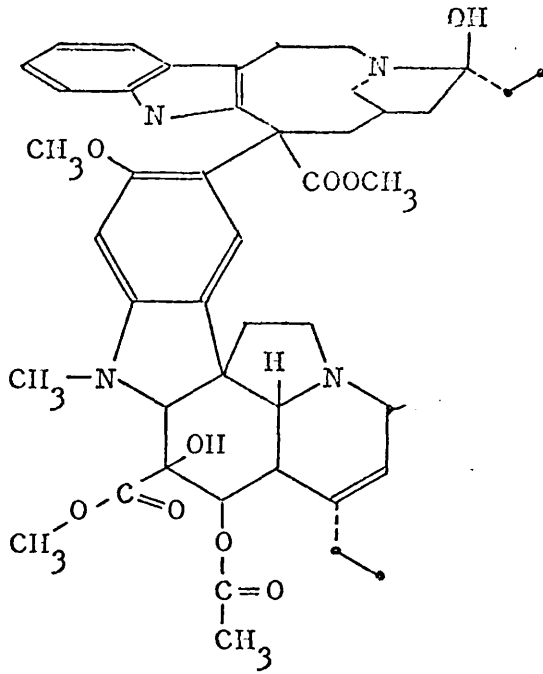
The formation of coiled-coils of protofilaments from tubulin <sup>e</sup>pretreated with leurosine or leurosidine, suggested that these drugs are acting on the lateral binding sites of tubulin, while the longitudinal sites are unaffected. The specific interaction of these drugs to the lateral sites of tubulin allows the formation of protofilaments but prevents the lateral association of these filaments into microtubules (Fig. 6.7 c & d). These two compounds when added to the polymerised microtubules, disrupt each microtubule at its ends, <sup>e</sup>releasing the constituent protofilaments due to their effect on the lateral sites between protofilaments. The released protofilaments would coil up into spiral structures as illustrated in Fig. 6.11. Single coils or ring-like structures were also detected. These effects are comparable to those observed by Kirschner et al. (1974) when assembled microtubules were treated with 2 mM-CaCl<sub>2</sub> or subjected to cold treatment at 4° for 24 h.

In their chemical structures (see Fig. 6.12) leurosidine and leurosine differ from vincristine and vinblastine in the catharanthine moiety; recent evidence Wilson et al., 1974), suggests that this moiety contributes to the biological activity of Vinca alkaloids. The other portion of these compounds consists of a vindoline moiety, which is biologically inactive, at least inasmuch as it is inactive in causing mitotic arrest in EHB hamster cells (Wilson et al., 1974). It is interesting that the difference in the positioning of the OH group in the catharanthine moiety of these compounds (see Fig. 6.12) could cause such a large variation in the mode of action on intact microtubules. Further study with analogues containing different

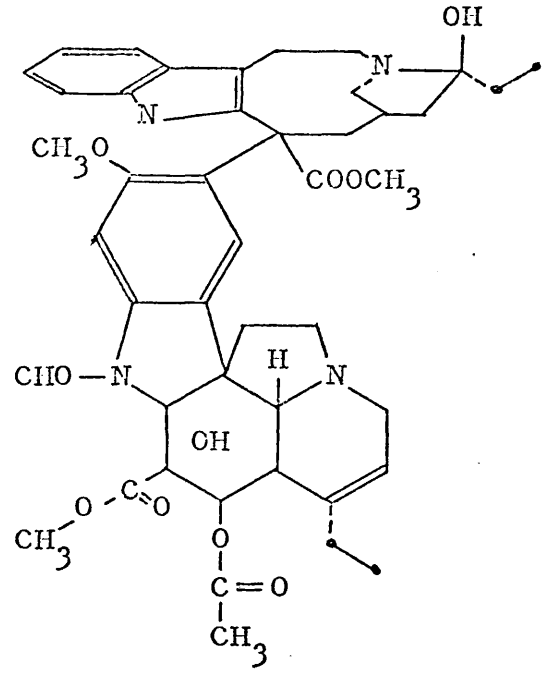
Diagrammatic representation of leucosine and leucosidine-induced fraying of reassembled microtubules

FIG. 6.11.

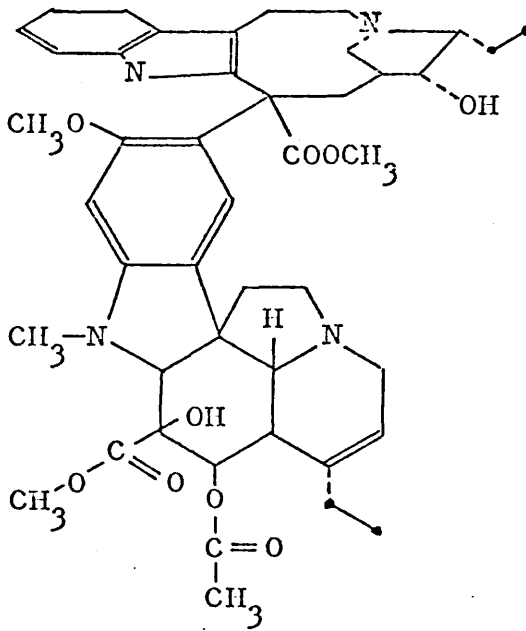




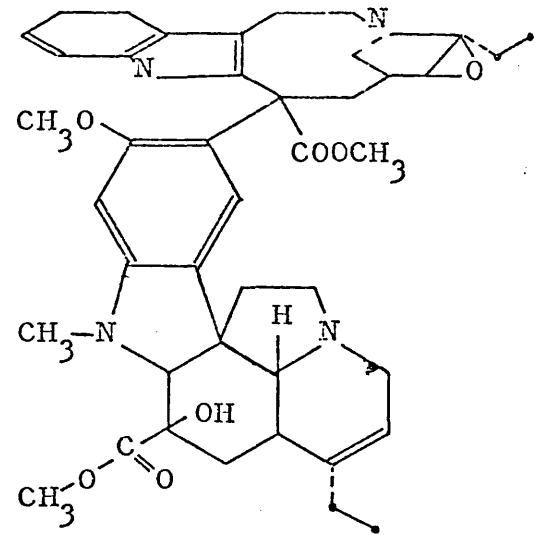
Vinblastine



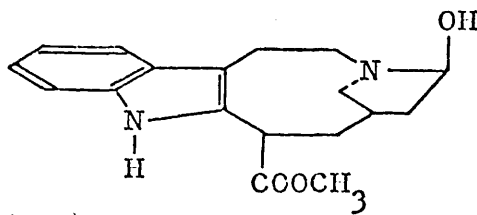
Vincristine



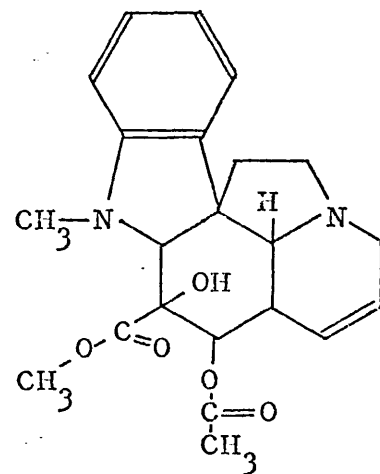
Leurosine



Leurosine



Catharanthine (active moiety)



Vindoline (inactive)

FIG.6.13.

Life-like structure seen during the in vitro growth of *Microthrix*.

---

magnification: 30K



magnification: 10K



See text, section 6.1. for details.

positioning and substitution of the OH group on catharanthine moiety could yield information of pharmacological importance.

The disassembly and assembly of microtubules in vitro not only can be used as a simple model for the study of direct effects of drugs on microtubular formation and structure, but also provides an opportunity for studying the physiological environment that would affect the dynamic equilibrium between the free and polymerised forms of tubulin (see Kirchner et al., 1974). The mechanisms that regulate the assembly and disassembly of microtubules has been a subject of great interest. In the absence of added drugs, disc-like structures have occasionally be observed in addition to intact microtubules (see Fig. 6.13.). These disc-like structures have approximately the same diameter as the microtubule.

Borisy & Olmsted (1972) have reported observing this same type of disc-like structures in a different reassembly system. They suggested that these disc-like elements are possibly nucleation centres, responsible for the initiation of microtubule assembly. Recently, using viscometric analysis, Kuriyama & Sakai (1974) have confirmed this suggestion. Our observation of these disc-like structures lends support to their claim. It would seem that the difficulty in obtaining evidence for such nucleation centres reproducibly may be related to the time of sampling for fixing and staining: indeed, the in vitro polymerisation of tubulin subunits into microtubules takes place very rapidly, i.e. within a few minutes. The possible relationship of the disc-like structures to the double rings observed by Kirchner et al (1974) will be further discussed in the final chapter.

Chapter VII      Discussion and Conclusion

- (a)      Purification of tubulin
- (b)      Colchicine Binding
- (c)      Nucleotide Binding
- (d)      Vincristine Binding
- (e)      Effects of drugs on binding properties of tubulin
- (f)      Conclusion

Chapter VII      Discussion and Conclusion

The results of studies on different aspects of foetal rabbit brain tubulin which were presented separately in Chapter 2 - 6 have been briefly discussed at the end of each of these Chapters. Some of the main findings will now be further discussed in a wider context.

(a)      Purification of tubulin

It was shown that tubulin could be purified from foetal rabbit brain by three main methods of purification previously used to isolate the protein from adult brain (Chapter III, section 3.2). However, a slight modification was required in order to maximise the yield of tubulin when the chromatographic procedure was employed. Thus, it was found that by raising the amount of ammonium sulphate used in the second precipitation step from 43 % to 60 % saturation, the yield of tubulin in the final preparation was consistently increased by about 15 % (Chapter III, 3.2), suggesting that foetal brain tubulin may differ in some structural aspect from adult brain tubulin.

It is not known whether foetal brain tubulin differs chemically from that found in adult brain, although Bamburg et al. (1973) have recently shown that the half-life for the loss of colchicine-binding activity during aging at 37°C in soluble extracts of chick embryo brain was greatest in the period between 5 - 9 days in ovo, and decreased rapidly as development continued. These authors suggested that the observed decrease in stability of colchicine-sites during development might be related to an alternation in the structure of the protein, or more indirectly



to the presence of an unidentified stabilising factor in embryonic extracts of brain.

In this connection, it may be relevant that the majority of the phosphorylated form of tubulin can be precipitated in the first ammonium sulphate fraction (0 - 32 % saturation), whereas a higher ammonium sulphate concentration is required to precipitate the relatively unphosphorylated form of tubulin during the second ammonium sulphate step (32 - 60 % saturation) (Murray & Froscio, 1971; Lagnado et al., 1975). It is therefore conceivable that foetal brain tubulin may contain less bound phosphate than adult brain tubulin and this could then account, at least in part, for the anomalous behaviour observed in the initial salt fractionation step during purification. Adult brain tubulin isolated by chromatography or by polymerisation contains up to 1 mole of protein-bound serine phosphate per mole of tubulin dimer (M.W. 110,000) (Reddington & Lagnado, 1973; Eipper, 1974; Lagnado et al., 1975), but the bound phosphate content of foetal brain tubulin remains to be determined.

Similar to other tubulin preparations, foetal rabbit brain tubulin was also absorbed onto DEAE-Sephadex, from which it was eluted by 0.5 - 0.8 M-NaCl (Chapter III, 3.2). Polyacrylamide gel electrophoresis under disaggregating conditions in the presence of urea provides a useful criterion for the identification of tubulin on the basis of its molecular weight: using this technique, foetal brain tubulin was found to comigrate with tubulin purified from adult brain in these different gel systems (see below).

Furthermore, it was shown that incubation of soluble extracts of foetal rabbit brain at 37°C in "assembly" buffer (see Chapter III, 3.2 & Chapter VI, 6.2.1) resulted in the polymerisation of tubulin into microtubules similar in every respect to those formed from soluble extracts of adult rabbit, rat and guinea pig brain as judged by examination of negatively stained samples under the electron microscope. On polyacrylamide gel electrophoresis, microtubular protein purified from foetal rabbit brain by two cycles of assembly-disassembly co-migrated with tubulin purified by the chromatographic method, and with tubulin purified from adult rabbit, rat or guinea pig brain by either method. In a continuous phosphate buffered system, it behaved as a single band with a molecular weight of about 56,000. Moreover, foetal rabbit brain tubulin was also separated into two closely related fractions, coinciding with the  $\alpha$  and  $\beta$  subunits of adult brain tubulin when electrophoresis was carried out in a discontinuous tris-glycine buffered system in the presence of SDS and urea (cf. Chapter III, 3.2.1).

The apparent molecular weights of  $\alpha$  and  $\beta$  subunits were found to be approximately 58,000 and 52,000, respectively. It is generally accepted that the molecular weight of the colchicine-binding dimer from pig brain gives values between 110,000 and 120,000, as judged by high-speed sedimentation equilibrium methods (which give Svedberg coefficient of 5.8 - 6.0 S) or by chromatography on calibrated Sephadex G200 columns (Weisenberg et al., 1968).

However, there is still some doubt as to whether the subunit

of microtubules is composed of a tubulin heterodimer ( $\alpha, \beta$ ) or a homodimer ( $\alpha\alpha, \beta\beta$ ), and hence the significance of the apparent difference in molecular weight found between the two subunits remains uncertain. For example, a recent report by Bibring & Baxandall (1974) has shown that the resolution of tubulin into  $\alpha$  and  $\beta$  subunits in SDS-polyacrylamide gels containing urea, depended on the amount of protein loaded. These authors showed that a gradual decrease in the amount of protein loaded was accompanied by a progressive disappearance of  $\beta$  subunits which could be detected by Coomassie Blue stain. On the other hand, Bryan (1974) showed that at high ionic strength and neutral pH, tubulin behaved as a single band and that running the gels in low ionic strength buffers, especially when alkaline (pH > 8) buffers were used, the  $\alpha$  and  $\beta$  subunits were easily separably. It was also shown that the apparent molecular weight of subunits remained constant (at neutral pH) at different ionic concentrations, but that the apparent molecular weight of the  $\alpha$  subunit appeared to decrease with increasing ionic strength (i.e. this corresponds to a higher mobility in SDS gels). From these and other data, Bryan (1974) deduced that both  $\alpha$  and  $\beta$  subunits might in fact have the same molecular weight of approximately 52,000 and that the apparent difference in molecular weight between the two subunits could be attributed to a charge effect. Indeed, it is known that the two polypeptides differ significantly in their amino acids composition (Bryan & Wilson, 1971). However, there may be an even higher degree of heterogeneity in tubulin than that associated with the presence of  $\alpha$  and  $\beta$  subunits, since it was shown that

tubulin chromatographically purified from adult brain (Feit et al., 1971b) gave four to five bands of almost equal staining intensity during isoelectric focussing in ampholine in the presence of 8 M urea. One obvious possibility to account for the added heterogeneity is that isoelectric focussing resolved phosphorylated and unphosphorylated species of the  $\alpha$  and  $\beta$  forms of tubulin.

In addition, foetal brain tubulin prepared by the chromatographic method always contained a minor component (MW ca. 42,000) migrating ahead of the main tubulin bands. This impurity, which accounted for less than 10 % of the staining, comigrated with rabbit muscle actin. It is interesting in this respect that tubulin partly purified from rat brain supernatant by precipitation with vinblastine also contains an actin-like protein (Fine & Bray, 1971). On the other hand, this component was not seen in tubulin preparations isolated from in vitro reassembled microtubules (see Chapter II, Fig. 2.4); instead, protein prepared by the polymerisation method was consistently found to contain 2-3 minor polypeptides of high molecular weight (> 200,000) which constituted 10 - 15 % of the total protein stained with Coomassie Blue. These minor components were occasionally detected in chromatographically purified tubulin. Although no accurate estimates of molecular weight for these components were made, recent studies by Burns & Pollard (1974) and Gaskin et al. (1974) have shown that proteins of similar size also copurified with tubulin isolated by several cycles of assembly-disassembly of microtubules from soluble extracts of dogfish, guinea pig, calf and pig brain. It now seems likely that <sup>the minor high</sup> molecular weight fractions which invariably copurify with

tubulin may represent the dynein-like ATPases which are thought to be associated with microtubules in situ (Gaskin et al., 1974; Burns & Pollard, 1974). Indeed, Reddington & Lagnado (manuscript in preparation) have recently shown that microtubular protein from guinea pig brain possesses a  $\text{Ca}^{2+}$ -activated ATPase activity which was tentatively attributed to the presence of the minor high molecular weight components observed. It may be relevant in this connection that these components were shown to be actively labelled in situ with  $\{^{32}\text{P}\}$  implying that they might represent phosphorylated intermediates associated with ATPase activity (Reddington, Tan & Lagnado, 1975). In addition, it is worth noting at this stage, that a similar pattern of protein labelling with  $\{^{32}\text{P}\}$  was seen when purified synaptic plasma synaptic membranes were incubated with  $\{\gamma^{32}\text{P}\}$  ATP: the tubulin-comigrating component and the dynein-like components present in purified synaptic membranes represented the main sites of protein bound  $\{^{32}\text{P}\}$  (see Fig. 3.15; Lagnado, Tan, Matus and Walters, Manuscript in preparation).

(b) Colchicine Binding

The colchicine-binding activity of purified tubulin varied according to the method of purification employed and to the amount of stabilising agent (e.g., GTP, sucrose or glycerol) present during the preparation. Although the yield of tubulin from foetal rabbit brain prepared by the chromatographic method was rather similar to that found for tubulin isolated by the polymerisation procedure (approximately 3 - 4 % of the protein present in the initial high-speed supernatant; or about 1 mg/ g of tissue wet weight), the colchicine-binding activity of tubulin which was purified by the latter method normally gave

higher values. This may be due to stabilising effects of GTP and glycerol which were present throughout the whole purification procedure. Conversely, the exposure of tubulin to high concentration of NaCl during the elution of protein from DEAE-Sephadex during the chromatographic purification caused the formation of inactive protein aggregates which resulted in an irreversible loss of colchicine-binding activity. This strong tendency for tubulin to form inactive aggregates in concentrated solution has been previously reported (Shelanski & Taylor, 1968). The colchicine-binding capacity of tubulin purified by the polymerisation procedure, gave values of approximately 0.6 mole/120,000 g of protein and after making corrections for thermal decay occurring during incubation at 37°C, this corresponded to about 0.8 mole/mole of tubulin dimer (M.W. 120,000), which is close to the values reported for adult pig brain (1 mole/tubulin dimer, see Weisenberg & Taylor, 1968) (see Table 3.4.b). A similar value was also found for adult guinea pig brain tubulin derived from in vitro reassembled microtubules (see Reddington, Tan & Lagnado, 1975).

It is generally assumed that tubulin preparations purified by the polymerisation procedure contain mainly the 6 S (M.W. 110,000) tubulin dimer species. However, Kirschner et al., (1974) recently pointed out that depolymerisation of the reassembled microtubules at low temperatures or in the presence of 2 mM Ca<sup>2+</sup> in fact gave rise to two forms of tubulin present in roughly equal concentrations. One form consists, indeed, of the 6 S tubulin dimers, whereas the other form, which can be seen as double ring-like structures

(ca. 430 Å outer diameter) by electron microscopy, behaved as a 30 S particulate during ultracentrifugation. When the 30 S tubulin aggregate was assayed for colchicine-binding activity, as it was found to bind colchicine one-tenth as effectively as the 6 S species, even in the presence of 1 M NaCl which caused complete dissociation of the 30 S particle into the 6 S dimeric components of tubulin. It was suggested that tubulin stabilised in the form of 30 S particles is important in initiating microtubule assembly, whereas the 6 S form which readily bound colchicine might represent a different type of tubulin, involved mainly in the elongation of tubular structures during assembly and not in the initiating step. The 6 S and 30 S forms of tubulin could not be distinguished after SDS-polyacrylamide gel electrophoresis. Therefore, in view of these findings, it is probable that only part of the protein present in the cold depolymerised microtubular preparations studied in the present work was responsible for most of the colchicine binding activity.

It now appears that the ring-like structures observed in depolymerised microtubule preparations by Kirschner et al. (1974) are similar to the disc-like elements which could be observed during assembly of microtubules in vitro (present work: see Chapter VI, Fig. 6.13). Such disc-like elements have been implicated as nucleating centres for initiating microtubule assembly (Borisy & Olmsted, 1972). The nature of the difference between the 6 S (high colchicine-binding) and the 30 S (low colchicine-binding) species of tubulin is unknown, though it has been observed that high molecular weight colchicine-binding aggregates of tubulin seen after sucrose gradient centrifugation are readily phosphorylated in situ (Reddington & Lagnado, 1973). This could signify that phosphorylation of

tubulin may be responsible for promoting the formation of stabilised 30 S aggregates.

In Chapter III (section 3.2.7), it was shown that sucrose protected the loss of both colchicine- and GTP-binding activities, apparently by preventing the formation of inactive aggregates. It seems likely that sucrose and glycerol protect the decay of these binding activities by stabilising the whole protein molecule in a dense medium, rather than by acting on the specific binding sites. The inclusion of GTP (100  $\mu$ M) in the buffers used during the chromatographic purification of tubulin considerably increased the colchicine binding activity of the final preparation, which bound approximately twice as much colchicine as tubulin purified in the absence of added GTP (see Chapter III, 3.3.2). The same concentration of GTP to tubulin may result in a more stable configuration of the protein, such as would also favour its assembly into microtubular structures, since it has been shown that GTP is required for the polymerisation process. However, it should be added that colchicine-binding sites appear to be masked in the assembled microtubule, since colchicine does not cause depolymerisation of in vitro reassembled microtubules (Shelanski et al., 1973; Borisy et al., 1973).

(c) Nucleotide Binding

In order to facilitate the study of the role of guanine nucleotides bound to the non-exchangeable (N) and exchangeable (E) nucleotide-binding sites of tubulin, a more convenient



radiometric assay using Millipore filtration techniques was developed and applied to study the nucleotide-binding properties of foetal rabbit brain tubulin. The data obtained from competition experiments in which the GTP-binding activity to the exchangeable site of tubulin was assayed in the presence of various guanine and adenine nucleotide derivatives, indicated that the hydrolysable nucleotide triphosphates, particularly GTP and ATP, are preferred substrates, while the non-hydrolysable nucleotide analogues GMPPCP and AMPPCP are very poor substrates for the binding reaction. These findings support recent evidence for hydrolysis of the  $\gamma$  phosphate group of GTP bound to the E site of tubulin (Borisy *et al.*, 1972). Berry & Shelanski (1972) have presented evidence for a transphosphorylation reaction occurring between GTP bound to the E site and GDP bound at the N site, and suggested that this reaction might be important in the assembly of microtubules. This interpretation is complicated by the new observations made in the present work, in which evidence for a distinct ATP-binding site(s) on tubulin has been presented; moreover, it was shown that the binding of ATP to tubulin was less specific than the binding of GTP (see Chapter III, 3.2.5). These observations have now been complemented by the work of Jacobs *et al.* (1974) who reported that although ATP could bind weakly to the E site, it was found to be a better substrate than GTP in the transphosphorylation reaction. The same authors also suggested that there is a third site on tubulin itself, or on another protein associated with it, which has a higher affinity for ATP than for GTP. No attempt has been made in the present work

to study the kinetic properties of the ATP-binding site(s) in any detail. However, it has also been reported that GTP can replace ATP in the phosphorylation of tubulin in vitro (Piras & Piras, 1974). It is therefore possible that the site which appears to bind ATP preferentially may be associated with the protein kinase activity associated with tubulin (Goodman et al., 1970). Alternately, it is conceivable that the minor high molecular weight components which copurify with tubulin could account for part at least of an ATP-specific binding activity.

(d) Vincristine Binding

The addition of 0.5 mM-vincristine to brain homogenates resulted in a dramatic redistribution of colchicine-binding protein from the soluble to the particulate fraction in both foetal brain and adult rat brain extracts (see Chapter III, Table 3.3, see also Lagnado & Lyons, 1971). This shows that foetal rabbit brain tubulin, like any other brain tubulin preparation, interacts with relatively low concentration of Vinca alkaloids to form aggregates which can be precipitated by high-speed centrifugation. However, besides causing a selective aggregation of tubulin, vincristine and vinblastine can also induce the precipitation of some other soluble proteins of brain (see, e.g. Fine & Bray, 1971). In addition, Wilson et al., (1970) have shown that relatively high concentrations of vinblastine (1 mM) also precipitated various structural proteins associated with membrane structures and they suggested that vinblastine induced the precipitation of these proteins through interactions with protein  $Ca^{2+}$ -binding sites. It has also been suggested that the formation of tubulin aggregates may be due to less specific hydrophobic interactions between Vinca

alkaloids and protein (Nimni, 1972). It is clear therefore that the Vinca alkaloids are less specific than colchicine in interacting with microtubular protein.

In order to study further the relative specificity of receptors for Vinca alkaloids in neural tissue, the subcellular distribution of vincristine- and colchicine-binding receptors was investigated. Preliminary studies on the binding of [ $^3\text{H}$ ] vincristine to purified tubulin from foetal rabbit brain showed that tubulin possessed two high-affinity binding sites; the binding constant for these sites were  $1.45 \times 10^5$  and  $9 \times 10^5$  litre/mole (data not shown). These values are in close agreement with those recently reported for chick embryo brain tubulin, using [ $^3\text{H}$ ]vinblastine (Wilson *et al.*, 1974). Using two different methods of subcellular fractionation of brain, it was found that in contrast to the enrichment of colchicine-binding protein in the soluble fraction, vincristine-binding receptors were mainly associated with the various particulate fractions obtained (i.e. nuclear, mitochondrial, and microsomal fractions (Chapter IV, Fig. 4.10a). Further subfractionation of the crude mitochondrial fraction showed that the fraction which was enriched in 'light' membranes gave the highest value for vincristine-binding activity, when this is expressed in terms of Relative Specific Activity (R.S.A.) (Chapter IV, Fig 4.11.b). The subfraction enriched in synaptic vesicles also gave high R.S.A. values for both vincristine and colchicine binding (see Chapter IV, Fig. 4.11.b). In comparing these data with those for colchicine-binding reported by Lagnado *et al.* (1971), it is evident that the pattern of distribution of vincristine-binding receptors in various subcellular fractions does not follow that seen for colchicine-binding protein. Thus, physiological processes which are affected by Vinca alkaloids may

not necessarily reflect an interaction of these drugs with microtubular protein. However, the observed enrichment of vincristine-binding receptors in the light membrane fraction would also be consistent with the view that neural tissue contains a significant pool of membrane-bound tubulin (Lagnado et al., 1971, 1972, 1975; see also Chapter III, section 3.5) which may be of functional importance. In a recent report, Hanbauer et al. (1974) demonstrated that vinblastine decreased the noradrenaline content of the adrenergic neurons by acting at the adrenergic synaptic terminals. Furthermore, the induction of the postsynaptic membrane of myoneural junction is delayed in the presence of colchicine and vinblastine (Jun<sup>o</sup>tunen, 1974). These observations inter alia, indicate that colchicine and Vinca alkaloids may disrupt neural function by acting via membrane-bound forms of tubulin. Other evidence that tubulin may be involved in cell membrane function comes from the work of Edelman et al. (1973) in which it was demonstrated that colchicine and Vinca alkaloids inhibited the mobility of concanavalin A receptors in lymphocyte membranes. Therefore the presence of tubulin as drug receptors in membranes and as structural components for maintaining the integrity of cell membranes may be of great physiological and pharmacological interest.

(e) Effects of drugs on binding properties of tubulin

After the preliminary finding that both vincristine and vinblastine stabilised the colchicine- and GTP-binding activities, of tubulin, other indole compounds which bear some structural similarities to the Vinca alkaloids were also tested for their effects on the binding activities of tubulin. In contrast

to vincristine and vinblastine, indole compounds such as reserpine, yohimbine, harmine and melatonin were found to inhibit the binding of colchicine to tubulin (Chapter V, 5.2.3). It has been reported (Wilson, 1970) and confirmed in the present work (Chapter V, 5.4) that vinblastine and vincristine prevent the thermal decay of colchicine-binding activity of tubulin seen during incubation of the protein at 37°C. In contrast, reserpine (100 µM) was found to accelerate the thermal decay of foetal brain tubulin and this effect of reserpine could be partially reversed by the addition of the same concentration of vincristine (see Chapter V, 5.4). This suggests that reserpine and possibly other indole compounds which inhibited colchicine-binding may promote a loss of colchicine-binding activity through some interaction with tubulin sites similar to those responsible for vincristine binding. Furthermore, it was also observed that reserpine and melatonin at concentrations between 100 - 500 µM induced turbidity in the tubulin solution. It is therefore suggested that these two compounds may induce the formation of tubulin aggregates which, however, unlike those induced by the Vinca alkaloids do not retain their colchicine-binding activity. This would imply that the conformational change induced by reserpine and melatonin differ from those occurring in the presence of the Vinca alkaloids.

It is known that Vinca alkaloids induce the formation of highly ordered paracrystalline aggregates of tubulin both in vivo and in vitro (Bensch & Malawista, 1969; Bunt, 1973). The crystalline structures formed in intact cells in the presence of these drugs have been isolated and shown to bind colchicine and GTP (Bryan, 1974). To my knowledge, however, there is no published evidence indicating that either reserpine or melatonin

can induce similar crystalline aggregates of tubulin in vivo or in vitro. The nature of the tubulin aggregates responsible for the turbidity observed in tubulin solutions containing reserpine or melatonin (Chapter V) remains to be investigated. However, the fact that such aggregates do not apparently contain active colchicine binding sites might indicate that either the sites are 'denatured' or that they are masked as a result of tubulin-tubulin interactions similar to those occurring, for example, during the polymerisation of the protein into microtubular structures. Indeed, it has been shown that intact microtubular structures do not bind colchicine and this can be interpreted as signifying that the colchicine-binding site in microtubular polymers are masked.

Vinca alkaloids and methoxyindoles including reserpine, harmine, melatonin, 5-methoxyindole and serotonin were all found to increase the GTP-binding activity of tubulin. Harmine only increased the binding activity at high concentrations (500  $\mu\text{M}$ ) while yohimbine, the only compound tested lacking a methoxy group on the indole ring, had no effect on GTP-binding (Chapter V, 5.3), even though it was a potent inhibitor of colchicine binding. It would thus appear that a methoxy substitution is essential for causing an apparent increase stabilisation of GTP-binding sites. The effects of these compounds on the binding activities of tubulin cannot be easily explained on the basis of their similarities in chemical structure. However, Margulis (1973) recently showed that a number of antimitotic alkaloids in which a methoxy group on an aromatic ring is approximately 7 Å away from an electron-negative atom bound to hydrogen (i.e. -NH or -OH group) affect

the regeneration of Stentor ciliated oral membranelar band which consists of microtubular structures. It seems that most indole compounds except yohimbine which have been found to affect one or both the binding activities of tubulin, to some extent contain the requirement stated in Margulis's hypothesis regarding the structural specificity of anti-microtubular drugs. Other compounds tested in the present work, such as trimethoxybenzoic acid, D-lysergic acid, mescaline, amphetamine, tetrabenzine, dimethyltryptamine and tryptamine, which do not contain a methoxyindole moiety, were found to have no significant effect on the binding properties of tubulin.

As mentioned above, it is conceivable that the observed effects of methoxy indole derivatives on the binding properties of brain tubulin may be related to the ability of these compounds of inducing the formation of tubulin aggregates, possibly even microtubular structures. Thus, any compound which would induce the aggregation or polymerisation of tubulin might be expected to decrease colchicine-binding activity by reducing the number of free tubulin dimers capable of binding colchicine. Indeed, it has been shown that microtubules do not interact with colchicine directly (Shełanski et al., 1973; Borisy et al., 1973). Conversely, compounds which disrupt or depolymerise microtubular aggregates or structures would tend to increase the colchicine-binding activity by increasing the population of colchicine-binding tubulin dimer. At the same time, since GTP promotes the polymerisation of tubulin into microtubules, during which the nucleotides may be bound more firmly to the protein, it is conceivable that drugs which increase and/or stabilise the binding of GTP to tubulin (e.g., the methoxy-indoles tested)

might also promote microtubule assembly. These considerations would signify that the effects of methoxy indoles such as reserpine in inhibiting colchicine-binding and increasing GTP-binding, might be secondary to an effect on the state of aggregation of the protein. This possibility was explored by testing some of these drugs for their effects on the assembly of microtubules in vitro.

In this work, the effects of drugs on the assembly of microtubules in vitro was assessed qualitatively by electron microscopy. As might have been expected, the antimitotic drugs colchicine, vincristine, leurosine and leurosidine were all found to prevent the formation of normal microtubules. Some of the less potent Vinca alkaloids (e.g. leurosidine and leurosine) induced the formation of coiled protofilaments. Reserpine seemed to produce an accelerating effect on the process of assembly, resulting in an abundance of 'normal' microtubules. (Chapter VI, 6.3.2). Melatonin induced the appearance of 'curly' or flattened regions in otherwise 'normal' microtubules (see Chapter VI, 6.3.2). It is suggested that this malformation of microtubules may also be due to an accelerating effect exerted by melatonin on the process of polymerisation.

It was recently observed that when polymerisation in vitro of guinea pig brain microtubules was carried out at high concentration of protein, the assembly process occurred more rapidly (Shelanski et al., 1973). Under these conditions, some abnormal polymerised microtubules also observed in which regions of otherwise normal microtubules contained flattened stretches of protofilaments arranged in a sheet-like structure. One interpretation of Shelanski's observation is that during very rapid assembly of microtubular protein, error in the



polymerisation process would be 'frozen' into the structure due to the presence of the potent stabilising agent, glycerol. Indeed, no such abnormal features were observed when polymerisation took place even at high protein concentration, in the absence of glycerol. (In the absence of glycerol, microtubules would be in dynamic equilibrium with free tubulin subunits and errors in assembly could be more readily 'corrected' by exchange of subunits) By analogy, it may be supposed that the abnormality seen in the presence of melatonin could result from an accelerating effect of this drug on the assembly process which was studied at relatively low concentrations of protein. Alternatively, it is conceivable that melatonin may itself be incorporated into the growing microtubule without thereby preventing the assembly process, but rather causing some small localised conformational or chemical changes which result in an interruption of the normal assembly process. This possibility would be consistent with the results of Margulis (1973) who showed, for example, that microtubule regeneration in Stentor was prevented by colchicine, but that melatonin counteracted the effect of colchicine. Thus melatonin could be viewed as binding to sites similar to those normally occupied by colchicine, but in doing so, would not prevent the polymerisation of microtubular protein subunits. It should be stressed, however, that in Margulis's work that microtubule assembly was measured indirectly in terms of the regeneration of oral membranelar band which was assessed semiquantitatively by light microscopy. It is not known, therefore, whether microtubular regeneration in the presence of melatonin in Stentor experiments contained any regions of distorted appearance or irregularities.

These effects seen with melatonin open up the possibility of

a hormonal control on the assembly of microtubules in vivo. Indeed, it has been reported recently that another hormone, nerve growth factor, besides causes selective aggregation of tubulin in the high-speed supernatant of mouse brain, it also induces the outgrowth of neurites of cultured human neuroblastoma cells which results in an increase of colchicine-binding activity in extracts from these cultures (Calissano & Cozzari, 1974; Kolber et al., 1974). It is clear, however, that more direct quantitative investigations on the effects of these hormones on the assembly of microtubules in vitro using, for example, viscometry or flow birefringence techniques are required before the qualitative data obtained by electron microscopy can be properly assessed.

When added to assembled microtubules, these drugs behaved slightly differently. Whereas reserpine and melatonin did not appear to affect the appearance of reassembled microtubules, the Vinca alkaloids split the microtubules at both ends; the frayed ends apparently gave rise to coiled-coils of protofilaments (see Chapter VI, section 6.3.3.). Therefore, it seems likely that Vinca alkaloids act on the lateral bonds between protofilaments preventing their normal association to form microtubules. These results on the effects of Vinca alkaloids would seem to fit in rather well with the observations of Wisniewski et al. (1968) who showed that after subarachnoid injection of vinblastine and other mitotic spindle inhibitors, an accumulation of 'neurofilaments'

was detected in the neurons of spinal cord anterior horn cells. It should be pointed out that leurosine and leurosidine were later found to be the least potent amongs the Vinca alkaloids tested in their effects on the binding<sup>of</sup> colchicine and GTP to partly purified preparation of tubulin (new data not included in Results section) and this is in keeping with the limited ability of these compounds in disrupting reassembled microtubules (Chapter VI, 6.3.3.). Vincristine, which is more potent in increasing the colchicine- and GTP- binding activities of tubulin, completely disrupted the assembled microtubules (Chapter VI, 6.3.3.).

(5) Conclusions

From the foregoing discussion, it is evident that a well-characterised preparation of microtubular subunit protein isolated from foetal rabbit brain exhibits all the characteristic properties of tubulin prepared from adult mammalian brain. It seems clear that a number of neurotropic drugs which bear some structural similarities to the potent antimetabolic drugs (colchicine, vincristine) can interact not only with tubulin present in crude soluble and particulate extracts of brain, but also with the highly purified protein. In particular, it was shown that relatively low concentrations of several well-known methoxy-indole alkaloids markedly stabilised the GTP-binding activity of tubulin and, in contrast to the Vinca alkaloids, in some way inhibited colchicine-binding activity.

These drugs did not, however, appear to inhibit the assembly of microtubules in vitro under the conditions tested; this<sup>is</sup> in marked contrast to the obvious inhibitory effect on antimetabolic drugs on this process. Indeed, if anything, it was shown that reserpine and melatonin may even enhance the reassembly process in vitro. There is no counterpart for this observation from in vivo experiments (e.g., accumulation of microtubules in reserpinised animals) as judged by a survey<sup>e</sup> of recent literature concerning the effects of reserpine in vivo, although some authors have reported an accumulation of intranuclear filamentous bundles, possibly of microtubular origin, in certain neurones of reserpinised cats (Seite, 1970).

Obviously, in the case of reserpine and other psychotropic

drugs studied in this thesis, their most well-known short-term effects concern the regulation of monoamine storage and metabolism in central nervous system, and very few detail<sup>ed</sup> ultrastructural studies have been reported with animals treated with these drugs. On the basis of the present results, the lack of evidence for any effects of these psychotropic drugs on microtubules in vivo does not, of course, signify that they do not occur and clearly, this question would warrant further carefully controlled studies. The fact remains that a definite class of structurally related psychotropic drugs can interact with microtubular protein in vitro.

One limitation of the present work which restricts further discussion concerning the possible involvement of microtubules as a site of action for 'anti-tubulin' drugs, when their pharmacological effects on the nervous system are being investigated, is that this work was mainly concerned with the effects of these drugs on the soluble form of microtubular protein. It is well-known that reserpine, for example, interacts with membrane components of aminergic nerve-endings, and it would be useful to know whether this interaction involves the membrane-bound forms of tubulin which have been tentatively identified in isolated synaptosomal junctional membranes. In this connection, it is worth noting that a number of recent studies have clearly demonstrated a disruption of synaptic function by antimitotic alkaloids in in vivo experiments as well as in isolated neuromuscular junction preparations (see Chapter I, 5, Hormone and transmitter release). It seems reasonable to assume that the observed inhibition of synaptic function of Vinca alkaloids is due to a direct action of these drugs with some membrane-associated components and not to an indirect action via a block of

axoplasmic transport.

With regard to the unique effects of melatonin, a naturally occurring methoxy-indole, on brain microtubular systems, which were reported in this thesis, it should be mentioned that the mode of action of this compound within the nervous system appears to be totally unknown.

REFERENCES

- Adelman, M.R., Borisy, G.G., Shelanski, M.L. & Taylor, E.W. (1968) Fed. Proc. 27, 5, 1186.
- Alberty, R.A. (1961) The Enzymes, 5, 531.
- Allande, J.E., Seeds, N.W., Conway, T.W. & Weissbach, H. (1967) Proc. Nat. Acad. Sci. 59, 1566.
- Allende, J.E. & Weissbach, H. (1967), Biochem. Biophys. Res. Commun. 29, 82.
- Arnold, J. (1966) Biol. Bull. 131, 383.
- Asakura, S., Kasai, M. & Oosawa, F. (1960) J. Polymer. Sci. 44, 35.
- Bamburg, J.R., Shooter, E.M. & Wilson, L. (1973). Biochem. 12, 8. 1476.
- Banerjee, S., Kerr, V., Winson, M., Kellher, J.K. & Margulis, L. (1972), J. Protozool. 19, 1. 108.
- Banks, P., Mayor, D., Mitchell, M. & Tomlinson, D. (1971) J. Physiol. 216, 625.
- Beer, C.T. (1955), Brit. Empire Cancer Campaign, 33rd Ann. Rept. 487.
- Beer, C.T., Wilson, M.L. & Bell, J. (1964), Can. J. Physiol. Pharmacol, 42, 1.
- Benitez, H.H., Murray, M.R. & Chargaff, E. (1954) Ann. N.Y. Acad. Sci. 163, 731.
- Bensch, K.G. & Malawista, S.E. (1969), J. Cell Biol. 40, 95.
- Bergmeyer, H.U. (1963) in Methods of Enzymatic Analysis, Academic Press.
- Berry, R.W. & Shelanski, M.L. (1972), J. Mol. Biol. 71, 71.
- Bibring, T. & Baxandall, J. (1974) Exp. Cell Res. 86, 120.
- Borisy, G.G. & Taylor, E.W. (1967) J. Cell. Biol. 34, 525.
- Borisy, G.G. & Olmsted, J.B. (1972) Science 177, 1196.
- Borisy, G.G., Olmsted, J.B. & Klugman R.A. (1972) Proc. Nat. Acad. Sci. 69, 10, 2890.
- Borisy, G.G., Olmsted, J.B. Marcum, J.M. & Allen, C. (1973) Fed. Proc. 33, 2, 167.
- Bradford, H. (1969), J. Neurochem. 16, 675.
- Bray, G. (1960) Anal. Biochem. 1, 279.

- Bryan, J. (1971) J. Exp. Cell Res. 66, 129.
- Bryan, J. & Wilson, L. (1971) Proc. Nat. Acad. Sci. 8, 8, 1762.
- Bryan, J. (1972) Biochem. 11, 2611.
- Bryan, J. (1974) Fed. Proc. 33, 2, 152.
- Burns, R.G. & Pollard, T.D. (1974) FEBS Lett. 40, 2, 274.
- Bunt, A.H., (1973) Brain Res. 53, 29.
- Burton, P.R. & Fernandez, H.L. (1973) J. Cell. Sci. 12, 567.
- Calissano, P. & Cozzari, C. (1974) Proc. Nat. Acad. Sci. 71, 5, 2131.
- Cancilla, P.A. & Barlow, R.M. (1966) Acta. Neuropath. 6, 251.
- Clarke, J.B. & Nicklas, W.J. (1970) J. Biol. Chem. 245, 4724.
- Dahlstrom, A. (1968) Eur. J. Pharmacol, 5, 111.
- Dahlstrom, A. (1971) Phil. Trans. Roy. Soc. Ser. B. 261, 325.
- De Belleruche, J. (1973) Ph.D. thesis University of London.
- Deysson, G. (1968) Int. Rev. of Cytology, 24.
- Edelman, G., Yahara, I. & Wong, J. Proc. Nat. Acad. Sci. 70, 5, 1442.
- Eigsti, O.J. & Dustin, P. (1955) in Colchicine - in Agriculture, Medicine, Buology & Chemistry, Iowa State College Press.
- Ellman G.I., Courtney, J.D., Andres, J.R. & Featherstone, R.M. (1961) Biochem. Pharmacol. 1, 88.
- Eipper, B. (1972) Proc. Nat. Acad. Sci. 69, 2283.
- Eipper, B. (1974) J. Biol. Chem. 249, 5, 1395.
- Eipper, B. (1974) J. Biol. Chem. 249, 5, 1407.
- Eng, L.F., Pratt, D. & Wilson, L. (1974) Neurobiolo. 4, 301.
- Ertel, R., Brot, N., Redfield, B., Allende, J.E. & Weissbach, H. Proc. Nat. Acad. Sci. 59, 861.



- Evans, E.A., Sheppard, H.C. & Turner, J.C. (1970)  
J. Labelled Compounds, 6, 1, 76.
- Fairbanks, G. Levinthal, C. & Teeder, R.H. (1965 Biochem.  
Biophys. Res. Commun. 20, 343.
- Eit, H. & Barondes, S.H. (1970) J. Neurochem. 17, 1355.
- Feit, H., Dutton, G., Barondes, S.H. & Shelanski, M.L.  
(1971a) J. Cell. Biol. 51, 138.
- Feit, H., Slusarek, L. & Shelanski, M.L. (1971b)  
Proc. Nat. Acad. Sci. 68, 2028.
- Fine, R. & Bray, D. (1971) Nature 234, 115.
- Friedman, M.H. (1971) J. Cell Biol. 49, 916.
- Frigon, R.P. & Lee, J.C. (1972) Arch. Biochem. Biophys.  
153, 587.
- Gaskin, F., Kramer, S.B. Cantor, C.R., Adelstein, R. & Shelanski,  
M.L. (1974) FEBS Lett. 40, 2, 281.
- Goldman, R. (1971) J. Cell. Biol. 51, 752.
- Goodman, D., Rasmussen, H., DiBella, F. & Guthrow, C. (1970)  
Proc. Nat. Acad. Sci. 67, 652.
- Gilbert, D. (1975) Personal Communication to J.R. Lagnado.
- Gray, E.G. & Pumphrey, R.J. (1958) Nature, 181, 618.
- Gray, E.G. & Guillery, R.W. (1966) Int. Rev. Cytology, 19, 111.
- Greenices, H.F., McIntyre, R.W. & Beer, C.T. (1968) J. Med.  
Chem. 11, 254.
- Haga, T., Abe, T. & Kurokawa, M. (1974) FEBS. Lett. 39, 3, 291.
- Hakala, M.J., Glaid, A.J. & Schwert, G.W. (1956), J. Biol.  
Chem. 221, 191.
- Hanbauer, I., Jacobowitz, D.M. & Kopin, I.J. (1974), Brit.  
J. Pharmacol, 50, 219.
- Hepler, P., McIntosh, J.R. Cleland, S. (1970) J. Cell. Biol.  
45, 438.

- Hirano, A., Dembitzer, H.M. Kurland, L.T. & Zimmerman, H.M.  
(1968) *J. Neuropath, Exp. Neurol.* 27, 167.
- Huneus, F.C. & Davison, P.F. (1970) *J. Mol. Biol.* 52, 415.
- Inoue, S. (1952) *Biol. Bull.* 103, 316.
- Inoue, S. (1953) *Chromosoma*, 5, 487.
- Inoue, S. (1964) in *Primitive Mobile systems in Cell Biology*, 642. ed. Allen, R.D. & Kanuya, N. N.Y. Academic Press.
- Inoue, S. & Sato, H. (1967) *J. Gen. Physiol. Suppl.* 50, 259.
- Jacobs, M., Smith, H., & Taylor, E.W. (1974) *Mol. Biol.* 89, 455.
- James, K.A.C., Bray, J.J., Morgan, I.G. & Austin, L. (1970) *Biochem. J.* 117, 769.
- Jeffrey, P.L. & Austin, L. (1974) *Prog. in Neurobiol.* February, 1974, 207.
- Jones, D.H. & Matus, A. (1974) *Biochim. Biophys. Acta.* 356, 276.
- Juononen, J (1974) *Med. Biol.* 52, 164.
- Kalra, S.K. & Fragata, M. (1974) *FEBS. Lett.* 42, 1, 112.
- Karlsson, J.O. & Sjöstrand, J. (1969) *Brain Res.* 13, 617.
- Karlsson, J.O. & Sjöstrand, S. (1971) *J. Neurochem.* 18, 975.
- Katz, N.L. (1972) *Eur. J. Pharmacol.* 19, 88.
- Kidd, M. (1963) *Nature*, 197, 192.
- Kidd, M. *Brain* (1964) 87, 307.
- Kirschner, M.W., Williams, R.C., Weingarten, M. & Gerhart, J.C.  
(1974) *Proc. Nat. Acad. Sci.* 71, 4, 1159.
- Kolber, A.R., Goldstein, M.N. & Moore, B.W. (1974) *Proc. Nat. Acad. Sci.* 71, 10, 4203.
- Kristensson, K. (1970) *Acta Neuropath. (Berl)* 16, 54.

- Kristensson, K. & Olsson, Y. (1971) *Acta Neuropath.* (Berl) 19, 1.
- Kuriyama, R. & Sakai, H. (1974) *J. Biochem. (Tokyo)*, 75, 463.
- Kuriyama, R. & Sakai, H. (1974) *J. Biochem. (Tokyo)* 76, 651.
- Lagnado, J.R. & Lyons, C.A. (1971) *Biochem. J.* 126, 3. 9p.
- Lagnado, J.R., Lyons, C.A. & Wickremasinghe, G. (1971) *FEBS Lett*, 15, 3. 254.
- Lagnado, J.R., Lyons, C.A., Weller, M. & Phillipson, O. (1972) *Biochem. J.* 128, 95p.
- Lagnado, J.R., Tan, L.P. & Reddington, M. (1975) Presented at N.Y. Acad. Sci. Conference on the Biology of Cytoplasmic Microtubules, May 1974, In Press: *Ann. N.Y. Acad. Sci. Abstr. (1974)* in *Trans. N.Y. Academy Sci. Ser. II*, 36, 6, 605.
- Lacy, P.E., Howell, S.L., Young, D.A. & Fink, C.J. (1968) *Nature* 219, 1177.
- Laemmlli, U.K. (1970) *Nature*, 227, 680.
- Lampert, P., Blumberg, J.M. & Pentschew, A. (1964), *J. Neuropath, Exp. Neurol.* 23, 60.
- Lauffer, M. (1958) *Nature*, 181, 1338.
- Lowry, O., Rosebrough, N., Farr, A. & Randall, R.J. (1951) *J. Biol. Chem.* 193, 265.
- Ludueno, R. & Woodward, D. (1973) *Proc. Nat. Acad. Sci.* 70, 12, 3594.
- Maggiolo, C. & Haley, T.S. (1964) *Proc. Soc. Exp. Biol. Med.* 115, 149.
- Marantz, R., Ventilla, M. & Shelanski, M.L. (1969) *Science* 165, 498.
- Marantz, R. & Shelanski, M.L. (1970) *J. Cell. Biol.* 44, 234.
- Margolis, L. (1973) *Int. Rev. Cytology*, 34, 333.
- McIlwain, H. & Rodnight, R. (1962) in *Practical Neurochemistry*, Churchill Ltd., London.

- Metuzals, J. (1963) *J. Ultrastruct Res.* 8, 30.
- Mohrić, H. (1968) *Nature* 217, 1053.
- Mohri, H. & Shimonura, M. (1973) *J. Biochem. (Tokyo)* 209.
- Moran, D.T. & Varela, F.G. (1971) *Proc. Nat. Acad. Sci.* 68, 757.
- Munier, R. & Macheboeuf, M. (1951) *Bull. Soc. Chem. Biol.* 33, 846.
- Murray, A. & Froscio, M. (1971) *Biochem. Biophys. Res. Commun.* 44, 1089.
- Nazar, R.N., Lanford, H.G. & Wong, J. (1970) *Anal. Biochem.* 35, 305
- Nimni, M. (1972) *Biochem. Pharmacol.* 21, 485.
- Nagayama, A. & Dales, S. (1970) *Proc. Nat. Acad. Sci.* 66, 464.
- Ochs, S. (1971) *J. Neurobiol.* 2, 331.
- Oldham, K.G. (1968) *J. Labelled Compounds*, 4, 3, 127.
- Olmsted, J., Witman, G., Carlson, K. & Rosenbaum, J. (1971) *Proc. Nat. Acad. Sci.* 68, 2273.
- Olmsted, J.B. & Borisy, G.G. (1973) *Ann. Rev. Biochem.* 507.
- Owellen, R.J. & Donigian, D.W. (1972a) *J. Med. Chem.* 15, 894.
- Owellen, R., Owens, A. & Donigian, D. (1972b) *Biochem. Biophys. Res. Commun.* 47, 4, 685.
- Palmer, C.G., Livengood, D., Warren, A., Simpson, P.G. & Johnson, I.S. (1960) *Exp. Cell Res.* 20, 198.
- Perisic, M. & Cúenod, M. (1972) *Science*, 175, 1140.
- Piras, M.M. & Piras, R. (1974) *Eur. J. Biochem.* 47, 443.
- Poisner, A.M. & Bernstein, J. (1971) *J. Pharmacol. Exp. Ther.* 177, 102.
- Porter, K.R. (1966) in *Ciba Foundation Symposia on Principles of Biomolecular organisation*. 308. ed. Wolstenholme, G.E.W., & O'Connor, M. Churchill, London.
- Quinn, G.P., Shore, P.A. & Brodie, B.B. (1959) *Exp. Pharmacol. Ther.* 127, 103.

- Racker, E. (1950) *Biochem. Biophys. Acta.* 4, 211.
- Rebhun, L. & Sander, G. (1967) *J. Cell Biol.* 34, 859.
- Reese, T.S. (1965) *J. Cell Biol.* 25, 209.
- Reddington, M. & Lagnado, J.R. (1973) *FEBS Lett.* 30, 2, 188.
- Reddington, M. Tan, L.P. & Lagnado, J.R. (1975). Submitted for publication in *FEBS Lett.*
- Robert, F. & Cuénod, M. (1969) *Exp. Brain Res.* 9, 116.
- Sabatini, D.D., Bensch, K. & L. Barnett R.J. (1963) *J. Cell Biol.* 17, 19.
- Samson, F.E. (1971) *J. Neurobiol.* 2, 347.
- Satir, P. (1974) *Scientific America.* October, 1974, 46.
- Schmitt, F.O. (1968) *Neurosci. Res. Prog. Bull.* 6, 119.
- Schochet, S.S. Jr., Lampert, P.W. & Lindenberg, R. (1968) *Acta. Neuropath.* 11, 330.
- Seeds, N.W., Gilman, A.G., Amano, T.R. & Nirenberg, M.W. (1970) *Proc. Nat. Acad. Sci.* 66, 160.
- Shafritz, D., & Wllessbach, H. (1969) *Arch. Biochem. & Biophys.* 134, 638.
- Seite, R. (1970) *J. Ultrastruct. Res.* 30, 152.
- Shelanski, M.L. & Taylor, E.W. (1967) *J. Cell Biol.* 34, 549.
- Shelanski, M.L. & Taylor, E.W. (1968) *J. Cell Biol.* 38, 304.
- Shelanski, M.L., Albert, S., De Vries, G.H. & Norton, W.T. (1971) *Science* 174, 1242.
- Shelanski, M.L., Gaskin, F. & Cantor, C.R. (1973). *Proc. Nat. Acad. Sci.* 70, 3, 765.
- Shigewaka, Y., Roth, L., Píklaja, D. (1971) *J. Cell. Sci.* 8, 127.
- Sjöstrand, F.S. (1953) *J. Cell. Comp. Physiol.* 42, 45.
- Sjöstrand, J. (1970) *Brain Res.* 18, 461.
- Smith, D.S., Jarlfors, U. & Beránek, R. (1970) *J. Cell. Biol.* 46, 199.
- Smith, D.S. (1971) *Phil. Trans. Roy. Soc.* 261, 395.
- Stephens, R.E., Renard, F.L. & Gibbons, I.R. (1967) *Science*, 156, 1606.

- Stephens, R.E. (1971) in Subunits in Biological System. 5,  
Part A. 355, ed. Timasheff, S.N. & Fasman, G.D., Dekker.
- Soifer, D., Laszlo, A. & Scotto, J. (1972) Biochem. Biophys.  
Acta. 271, 182.
- Tao, M., Salas, M. & Lipmann, F. (1970) Proc. Nat. Acad.  
Sci. 67, 408.
- Taylor, E.W. (1965) J. Cell. Biol. 25, 145.
- Terry, R.D. & Pena, C., (1965) J. Neuropath. Expt. Neurol.  
24, 200.
- Terry, R.D. & Wisniewski, H. (1970) in Alzheimer's Disease  
& Related Conditions. 145. ed. Wolstenholme, G.E.W.,  
& O'Connor, M. Churchill, London.
- Temple, R., Williams, S.J., Wilber, J.F. & Wolff, J. (1972)  
Biochem. Biophys. Res. Commun. 46, 3, 1454.
- Tilney, L.G., Hiramoto, Y. & Marsland, D. (1966) J. Cell.  
Biol. 29, 77.
- Tilney, L. & Porter, K. (1967) J. Cell. Biol. 34, 327.
- Tilney, L. (1968) J. Cell Sci. 3, 549.
- Turkanis, S.A. (1973) Brain Res. 54, 324.
- Walters, B. & Matus, A. (1975) in Press. Trans. Biochem. Soc.
- Webb, E.C. (1964) Nature 203, 821.
- Weber, K. & Osborn, M. (1969) J. Biol. Chem. 244, 4401.
- Weisenberg, R.C., Borisy, G.G. & Taylor, E.W. (1968)  
Biochem. 7, 4466.
- Weisenberg, R.C. & Timasheff, S.N. (1970) Biochem. 9, 20, 4110.
- Weisenberg, R.C. (1972) Science, 177, 1104.
- Williams, J.A. & Wolff, J. (1970) Proc. Nat. Acad. Sci. 67,  
4, 1901.
- Wilson, L. & Friedkin, M. (1966) Biochemistry, 5, 2463.
- Wilson, L. & Friedkin, M. (1967) Biochemistry, 6, 3126.
- Wilson, L. (1970) Biochemistry, 9, 25, 4999.

- Wilson, L., Bryan, J., Ruby, A. & Mazia, D. (1970)  
Proc. Nat. Acad. Sci. 66, 3, 807.
- Wilson, L., Bamburg, J.R., Mizel, S.B., Grisham, L.M. &  
Creswell, K.M. (1974) Fed. Proc. 33, 2, 158.
- Wisniewski, H. & Terry, R.D. (1967) Lab. Invest. 17, 577.
- Wisniewski, H., Shelanski, M.L. & Terry, R.D. (1968)  
J. Cell. Biol. 28, 224.
- Wisniewski, H., Terry, R.D. & Hirano, A. (1970) J. Neuropath.  
Exp. Neurol. 29, 163.
- Wuerker, R.B. & Kirkpatrick, J.B. (1972) Int. Rev. of  
Cytology, 33, 45.
- Yamada, K.M., Spooner, B.S. & Wessels, N.K. (1970)  
Proc. Nat. Acad. Sci. 66, 1206.