

STUDIES ON THE DISTRIBUTION AND PROPERTIES
OF MICROTUBULAR PROTEINS IN NERVOUS TISSUE

by

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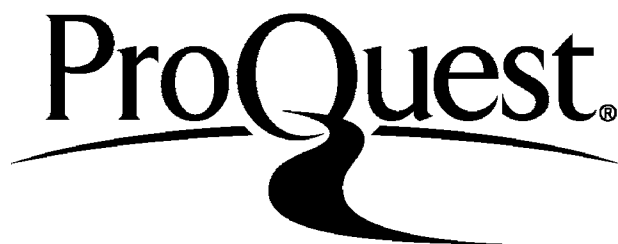
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ABSTRACT

The work reported in this thesis concerns investigation of the subcellular distribution of colchicine-binding activity (of presumed microtubular origin) in mammalian brain, and studies on the biochemical properties of colchicine-binding protein ('tubulin'). The results led to the following conclusions:

About half the CB-activity present in sucrose-^{containing}buffered homogenates of brain was consistently recovered in the particulate fraction obtained after high-speed centrifugation, and further investigations showed that CB-activity was widely distributed amongst the various subfractions obtained after gradient centrifugation of this pelleted material. The majority of the recovered particulate CB-activity was found to be associated with the fractions rich in synaptic membranes. Tubulin from this source appeared to represent a 'stabilised' form of CB-protein compared to that obtained from the high-speed soluble fraction, as judged from the results of experiments in which CB-activity was subjected to thermal decay under a variety of conditions. In addition, it was also shown that the tubulin isolated in particulate preparations was very tightly bound to membrane material, since it could not be readily solubilised by treatment with the detergent Triton X-100 or by sonication.

It was found, using partly purified tubulin preparations, that tubulin could act as a substrate for a cAMP-stimulated protein kinase that was closely associated with the protein, and that it bound cAMP. In addition, the antimitotic agent vincristine, which was found to stimulate CB-activity, also showed the remarkable property of almost selectively precipitating CB-protein from soluble extracts of brain, in the cold.

Finally, the previously inferred relation between neurotubular protein and CB-protein was proved when attempts made at isolating intact microtubules from brain homogenates prepared in various stabilising media were successful, and it was shown that these preparations were enriched in CB-activity.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

B.S.A.	-	(bovine) serum albumen
cAMP	-	adenosine 3':5'-cyclic monophosphate
C.B.	-	colchicine binding
conc.	-	concentration
mem.	-	membranes
MIT.	-	mitochondria
NEP.	-	nerve-ending particles ('synaptosomes')
partic.	-	particulate
ptn.	-	protein
R.S.A.	-	relative specific activity
S.A.	-	specific activity
SDS	-	sodium dodecyl (lauryl) sulphate
sol.	-	soluble
supt.	-	supernatant
TCA	-	trichloroacetic acid
VB	-	vinblastine
VC	-	vincristine

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Electron micrograph of rabbit brain microtubules, reassembled in vitro according to the polymerisation method of Shelanski et al (1973). Courtesy of Dr L P Tan. Magnification x 100,000.

CHAPTER ONE

Introduction and Literature Review

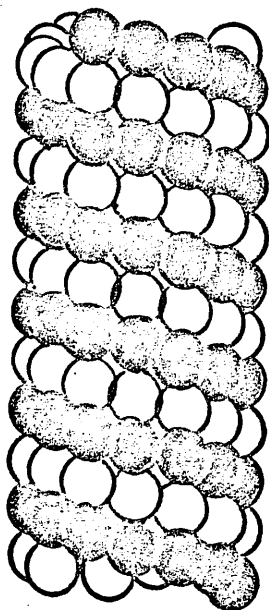
Microtubules are a class of long, tube-like organelles approximately 250 Å in diameter with a wall thickness of about 50 Å (see Fig. 1.1). Their existence was reported several times in the 1950's, but they were not encountered uniformly in cells until glutaraldehyde was introduced as a fixative (Sabatini et al, 1963). Microtubules have been found in all eukaryotic cells, and can be classified arbitrarily into two categories: stable and labile. Those found in the axons and dendrites of the central nervous system (Weurker and Kirkpatrick, 1972; Schmitt and Samson, 1968; Peters, 1970), in the mitotic apparatus of dividing cells (Inoué 1952) and in the cytoplasm of both animal and plant cells fall into the labile category; stable microtubules include those found in cilia and flagella.

Microtubules are implicated in a variety of cellular processes (see Porter, 1966; Adelman et al, 1968). From their occurrence, it has been suggested that microtubules are associated with the following functions:

- (a) Chromosome movements in cell division - in mitosis, a model has been proposed involving the sliding of microtubules, resulting in the separation of chromosomes. This is believed to be brought about by an interaction between microtubules that involves cross-bridges (McIntosh et al, 1969).

 - (b) Intracellular transport of materials - colchicine (see Fig. 1.2) and other antimitotic alkaloids (e.g. the Vinca alkaloids, see Fig. 1.3) that are known to disrupt microtubules, have also been shown to interrupt axoplasmic transport (Dahlstrom, 1968; Kreutzberg, 1969; Davison, 1970; Fernandez et al, 1971; Sjöstrand et al, 1970; Grafstein et al, 1970), although the precise nature of the mechanism involved remains to be clarified.
-
- (c) Maintenance of cell form and cellular motility - in erythrocytes (Gall, 1966) and blood platelets (Behnke and Zelander, 1967) microtubules seem to act as a cytoskeleton. It has been proposed (Gibbons and Gibbons, 1972) that the outer doublet microtubules of sperm flagella are a skeletal element important in maintaining cellular asymmetry and providing sites for attachment of the protein dynein (an ATPase) which functions as a force generator.

a)
Microtubule
surface
lattice



● α -subunit
○ β -subunit

b)
Cross-section
through a
microtubule

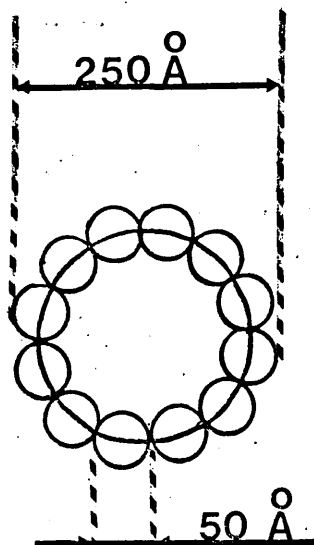
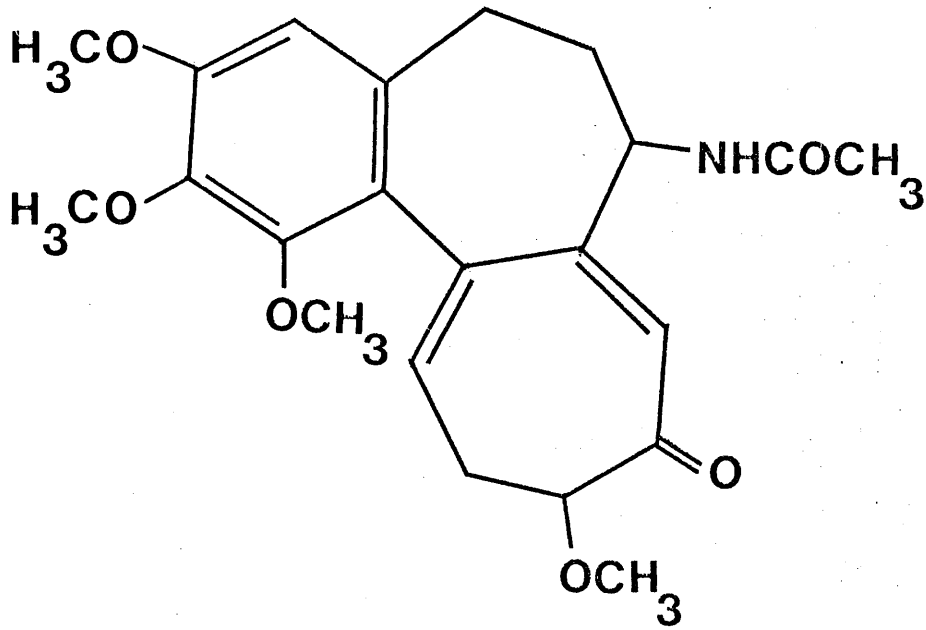


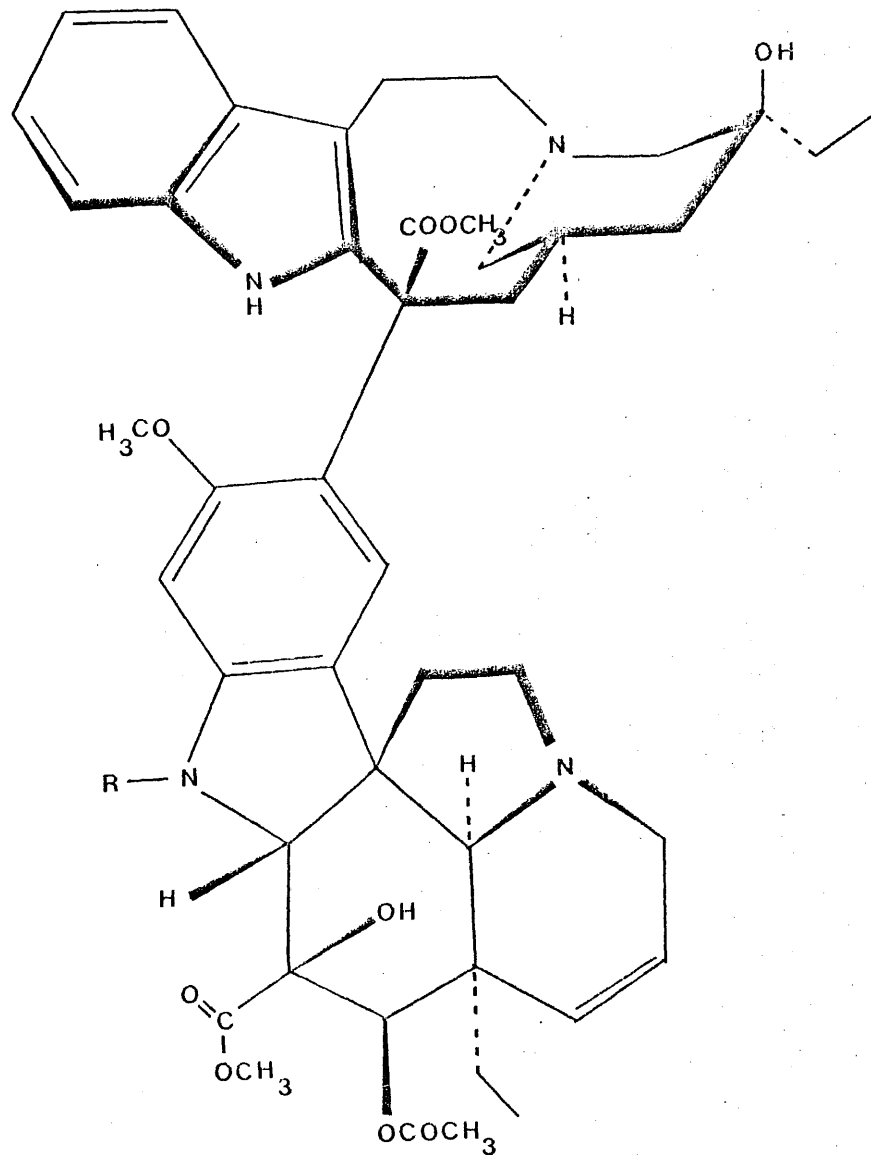
Figure 1-1

Figure 1.2



colchicine

Figure 1.3



vinblastine

R = CH₃

vincristine

R = CHO

From work on cultured nerve cells, it appears that microtubular systems play an important part in growth and differentiation (Prasad and Hsie, 1971). Microtubules probably also play an important role in the motile system of cilia and flagella (Bradfield, 1955; Jarosh, 1964). The mechanism of this movement is still unknown, although recently several aspects of these energy dependent processes have been investigated (Gordon and Barnett, 1967; Yanagisawa et al, 1968; Watts and Bannister, 1970; Stephens and Levine, 1970).

The evidence implicating microtubules in the processes of cell division, cell motility and the maintenance of cell shape led to interest in characterising the microtubule subunits and their process of assembly.

The first efforts to isolate microtubular protein were carried out with cilia and flagella, which contain microtubules as a main component, these being regularly arranged in a pattern of two ~~pairs~~ of microtubules in the centre surrounded by nine outer pairs of microtubules (Porter, 1966; Ringo, 1967; Gibbons and Grimstone, 1960). Gibbons (1963) and Renaud et al (1966) first reported the isolation of a protein from the outer fibres of cilia, which was later shown to bind colchicine (Borisy and Taylor, 1967a). A protein was also isolated from inner fibres which had similar physical and chemical properties, including ability to bind colchicine (Shelanski and Taylor, 1967; Gibbons and Renaud, 1968). The main component of the cilia protein had a sedimentation constant of 6.1S. in the ultracentrifuge, and molecular weight determinations gave a value of about 50,000 for the lowest molecular weight component of the protein. However, the average molecular weight was in excess of 100,000, indicating that the system contained dimers and higher aggregates of the smallest component. The outer pairs were also later shown to consist of A and B tubules, and these have been separately isolated (Stephens, 1970). The central-pair microtubule protein, extracted from sea-urchin sperm tails, was shown (Shelanski and Taylor, 1967) to bind $\frac{1}{2}$ -1 mole of colchicine per 100,000 grams; this protein-colchicine complex, when subjected to zone centrifugation, migrated with a sedimentation constant of 6S. Electrophoresis of the 6S material produced one prominent band on the gel, with a small amount of slower (higher molecular weight) material. Gibbons and Renaud (1968), who obtained a 4S and a 6S tubule protein from the central pairs of Tetrahymena cilia, showed these proteins to have molecular weights of 60,000 and 110,000 respectively; these proteins therefore appeared similar, in these respects, to those obtained from sperm tails.

Intact microtubules have been isolated from cilia, flagella, and from extracts of mitotic apparatus and mammalian brain in which the labile structures have been stabilised by the addition of ethanol or hexylene glycol (Stephens, 1968, and 1970; Witman et al, 1968; Kane, 1965; Kirkpatrick et al, 1970). However, due to the presence of the microtubule-preserving medium, colchicine-binding could not be demonstrated in these preparations.

Since the pioneering work of Inoué (Inoué and Sato, 1967), it has been generally accepted that labile microtubules are in a state of dynamic equilibrium, the assembled microtubules being in equilibrium with their depolymerised subunit protein ('tubulin') in the cytoplasm. It is believed that certain factors in the cell control the association and dissociation of the subunits. The most obvious example of the cyclic formation and breakdown of microtubules is during mitosis, when the spindle, which is composed of microtubules, forms at the beginning of the mitotic cycle, and is broken down when separation of the chromosomes is completed. These labile microtubules are readily disrupted by anti-mitotic agents such as colchicine and the Vinca alkaloids, and by low temperature or high hydrostatic pressure.

It has been known for some time that colchicine acts as a mitotic inhibitor by preventing the separation of divided chromosomes, and Mazia reported (1955) that colchicine interferes with the formation of spindle fibres during mitosis. Borisy and Taylor (1967 (a); 1967 (b)) demonstrated that colchicine ($[^3\text{H}]$ -labelled), when incubated with KB or HeLa cells in tissue culture, or with intact sea-urchin eggs, was bound non-covalently to a macromolecule present in the soluble fraction of homogenates. A similar binding reaction took place when extracts of the cells were incubated with colchicine. Wilson and Friedkin (1967) also demonstrated that colchicine binds to a soluble protein in grasshopper embryos. The binding was reversible, and did not involve chemical modification of the colchicine. It also appeared to depend on the protein being in its native state, since treatment with urea or perchloric acid produced almost complete release of the bound radioactivity. The kinetics of the reaction, both in vivo and in vitro, were consistent with the involvement of a single class of binding sites. Comparable results were obtained using the isolated mitotic apparatus from sea-urchin eggs, (which were found to be enriched several-fold in CB-protein), and with microtubule protein purified from this preparation. ~~CB-activity was found to correlate with the occurrence~~ CB-activity was found to correlate with the occurrence

of microtubules in cells and organelles: in cilia and sperm tails, microtubules make up the 9 + 2 array of filaments; in the mitotic spindle, microtubules form the framework; in brain, microtubules are present in large numbers in neuronal processes. Very little CB-activity was measured in liver, muscle, or kidney. This suggested that the binding sites were the subunit proteins of microtubules.

At present, most authors assume that colchicine binds specifically to the microtubular subunit protein. It has been suggested that the tubular structure is depolymerised by colchicine because of the formation of a complex of colchicine and microtubular subunit protein. It is supposed that the polymerisation of the subunits into microtubules is an equilibrium process, which can be shifted to favour monomer by complexing with colchicine, lowering the temperature, or increasing the pressure.

Isolated microtubule protein resembled muscle actin in amino acid composition (Shelanski and Taylor, 1968) and contained bound guanine nucleotides (Weisenberg *et al*, 1968). A variety of techniques have now been successfully applied to the purification of tubulin from various species, notably that of Weisenberg *et al* (1968) which involves ammonium sulphate fractionation of mammalian brain extracts, followed by ion-exchange chromatography on DEAE-Sephadex. Tubulin is highly acidic, and is eluted at relatively high ionic strength (0.5 - 0.8M) from the Sephadex. The CB-dimer isolated (M.W. 110,000) bound, uncompetitively, 1 mole of colchicine and 2 moles of GTP (1 of which was exchangeable); it contained, when purified, 0.5 - 0.8 mole of a mixture of GTP and GDP. Binding of GTP or colchicine appeared to stabilise the native configuration of the protein, as seen from sedimentation patterns and decay of binding activity. In terms of molecular weight, sedimentation constant, amino acid composition, binding of colchicine and GTP and behaviour during polyacrylamide electrophoresis, the protein appeared very similar to the subunit protein of cilia and flagella.

Bensch and Malawista (1969) discovered that crystals of tubulin could be induced to form using vinblastine, and Bryan (1971) isolated such crystals from sea urchin eggs. Tubulin obtained from intact structures, or the structures themselves, had the ability to bind colchicine, indicating identity between the tubulin isolated from intact microtubules and presumptive soluble tubulin. Wilson (1970) also showed that VB stabilised CB-activity by increasing the half-time of the decay process.

Vinblastine has also been used to precipitate tubulin in vitro from crude supernatants and from relatively pure preparations of tubulin (Bensch et al, 1969; Marantz et al, 1969; Olmsted et al, 1970; Wilson et al, 1970).

The molecular weight of tubulin has been determined by several methods including sedimentation equilibrium (Stephens, 1968 and 1970; Weisenberg et al, 1968) and gel filtration (Bryan and Wilson, 1971; Eipper, 1972; Wilson and Friedkin, 1967). Values for the soluble colchicine receptor lie between 110,000 and 130,000 daltons. Treatment of tubulin with denaturing agents (SDS, urea or guanine hydrochloride) results in loss of drug receptor activity and ability to polymerise. The loss of activity is accompanied by a decrease in molecular weight to about 50,000-60,000, demonstrating that tubulin is composed of at least two protomers.

Analysis of the protomers by polyacrylamide gel electrophoresis in the presence of urea and alkaline buffer shows that there are two distinct subunits (α - and β -, which has the greater mobility) (Bryan and Wilson, 1971). Under different conditions of electrophoresis (no urea, high ionic strength and neutral pH) (Weber and Osborn, 1969), the protomers behave as a single protein with a molecular weight of 52,000-55,000 daltons. The reasons for this anomalous behaviour are not yet known, but are believed to be concerned with interaction of SDS with the α -subunit, altering its mobility in the urea system with lowered ionic strength and raised pH.

The first direct evidence for chemical differences between the α - and β - tubulin subunits was obtained from the amino acid analysis on chick embryo brain tubulins (Bryan and Wilson, 1971). Significant differences were found in His, Cys, Ser, Ala, Met and Ileu. Partial sequence data (Luduena and Woodward, 1972) on the first 25 N-terminal residues of the α - and β -tubulins from sea urchin outer doublet and chick embryo brain cytoplasmic tubulins are now available. Both α -tubulins have the same sequence except for residue 25; the β -tubulins differ only at residue 7. However, the α - and β -tubulins differ in at least 12 positions, affirming the chemical non-identity suggested by the amino acid analyses.

It has recently been claimed (Outton and Barondes, 1969) that colchicine-binding protein accounts for nearly 40% of the soluble labelled protein in the brains of new-born mice after injection of 14 C-labelled leucine. Preliminary experiments in this laboratory indicated that a substantial proportion of colchicine-binding activity in pig brain homogenates was associated with the crude particulate fraction obtained after high-speed

centrifugation. A detailed investigation of the subcellular distribution of colchicine-binding activity in brain and a study of the properties of brain colchicine-binding protein was therefore begun. Several different aspects of brain tubulin chemistry were also investigated during the course of this work, in particular the reaction concerning the phosphorylation of tubulin. This reaction is thought, at present, to be at least part of the mechanism involved in microtubule function.

The object of this research was to answer certain questions about the structure and function of microtubules in nerve cells, which would hopefully lead to a better understanding of the role assigned to these organelles, and to conclusions of physiological value and importance.

The work reported on in this thesis was carried out during the period 1970-1973, only a few years after interest had been stimulated in microtubule biochemistry. Much more information has since appeared, during the preparation of this thesis; those areas of work which particularly extend this study are presented in the Discussion (Chapter 8).

CHAPTER TWO

General Methods

(a) Chemicals

All standard chemicals used in this work were of the highest analytical grade available. Radioactive chemicals were obtained from the Radiochemical Centre, Amersham, Bucks; specification of radiochemicals and details of the preparation of solutions containing them are given in the relevant sections of the text. Radioactive chemicals were stored at -20°C ; colchicine was additionally kept in the dark. Cold colchicine (obtained from Koch-Light, Colnbrook, Bucks) and radioactive colchicine (ring C-methoxyl tritiated) were checked for purity by thin-layer chromatography as described in section (i) of this chapter.

Nucleotides obtained from Sigma were the highest grade, and were used without further purification after checking their purity by thin-layer chromatography according to the method of Tao and Lipmann (1969), on cellulose-polyethyleneimine impregnated foils using acetic acid/LiCl solvent. Any batches containing significant amounts of impurity were not used.

Vinblastine and vincristine, a gift from Eli Lilly Co, were checked for purity by thin-layer chromatography on silica gel using ethanol solvent, as described in Southern Research Institute (1971).

(b) Preparation of tissue extracts

Brains from adult rats and guinea pigs were removed after the animals had been etherised then decapitated. The tissue was quickly transferred to filter paper moistened with ice-cold homogenising medium (see section (c) of this chapter). A 10% homogenate was made by adding 9 parts of ice-cold buffered sucrose, and the material was homogenised in a motor-driven Teflon-in-glass homogeniser (clearance 0.003-0.005"), at top speed, for 10 up-and-down strokes. For simple distribution studies, the homogenate was centrifuged at 4°C in an MSE superspeed 40 ultracentrifuge for 1h at $10^5 \underline{g}$ av. A soluble and a particulate fraction were obtained; the supernatant was decanted, and the pellet was resuspended in homogenising buffer by dispersal in a Teflon homogeniser by hand.

(c) Buffers

Tissue was usually homogenised in a 0.32M sucrose solution containing 1mM (or 10mM) sodium phosphate buffer, pH 6.8, and 1mM (or 10mM) MgCl_2 . This was prepared by mixing equal volumes of 0.1M solutions of disodium hydrogen phosphate and sodium dihydrogen phosphate, to give a stock

solution of 0.1M sodium phosphate buffer pH6.8. $MgCl_2$ (1M stock solution) was added to the appropriately diluted stock phosphate buffer solution, and the pH was adjusted to 6.8 when necessary. (This buffer will be referred to as P-Mg buffer.)

(d) Measurement of colchicine-binding (CB-) activity

(i) Incubation

Standard assays to measure CB-activity were carried out by incubating protein samples (containing up to 1mg protein/ml of incubation mixture) with [3H]-colchicine in a 37° water bath, in the dark, for 1.5h. (Dark conditions are necessary to prevent photo-oxidation of the colchicine to isomers which do not bind to CB- proteins. (See Wilson and Friedkin, 1966) The incubation mixtures were contained in a final volume of 0.6ml in 10mM P-Mg buffer and the final [3H]-colchicine concentration was 2.5 μM . After incubation, the samples were placed on ice for 5 minutes to terminate the binding reaction which is very slow in the cold. The radioactivity due to protein-bound [3H]-colchicine was usually determined by the DE81 filter disc assay as described below. Under these conditions, it was found that colchicine binding reached a maximum at 1.5h and was linear with respect to protein concentration. (See Chapter 5, Results section (a)).

(ii) Assay

Whatman DE81 filter discs (25mm diameter) were numbered with a pencil for identification and were placed on upturned screw caps in a petri dish placed on ice. They were moistened with ice-cold 10mM sodium phosphate buffer pH 6.8 (prepared by diluting stock buffer - see section (c)), and aliquots of the incubation mixtures were pipetted on (0.1ml/disc), and allowed to absorb for 5 minutes. The unbound colchicine was washed off the discs as follows: the discs were placed in a beaker containing ice-cold 10mM sodium phosphate buffer pH 6.8 (30-40ml buffer/disc) and were allowed to stand on ice for 5 minutes, with intermittent stirring. The buffer was decanted, an equal volume of fresh ice-cold buffer was added and the discs were left for a further 5 minutes. A total of five separate buffer washes was found to be sufficient to remove the unbound colchicine from the discs, as judged by measuring the amount of radioactivity in the washings (See Fig. 2.1). The discs were then briefly blotted on tissue, and placed separately in 5ml of Bray's scintillation fluid (See section (g), this chapter) in a radioactive counting vial. The

Figure 2.1

Removal of unbound colchicine from DE81 discs in the disc assay of CB-activity

0.1ml samples containing protein bound to [^3H]-colchicine and free colchicine were allowed to absorb on each disc at 4 $^{\circ}$. Each disc was then placed in 30ml ice-cold 10mM sodium phosphate buffer, pH 6.8, and was allowed to stand for 5 minutes to elute the free colchicine. 0.1ml samples of the buffer were taken (in triplicate) and were each placed in 5ml Bray's scintillation fluid in a radioactive counting vial, and the amount of radioactivity present in the sample was measured. The buffer was decanted from the discs, a further 30ml of fresh buffer was added, and the procedure was repeated until a total of 5 washes had been performed. The background radioactivity in each vial of scintillation fluid prior to addition of the sample was approximately 20cpm. This has not been subtracted from the results.

cpm/0.1ml
buffer

1500

1000

500

1

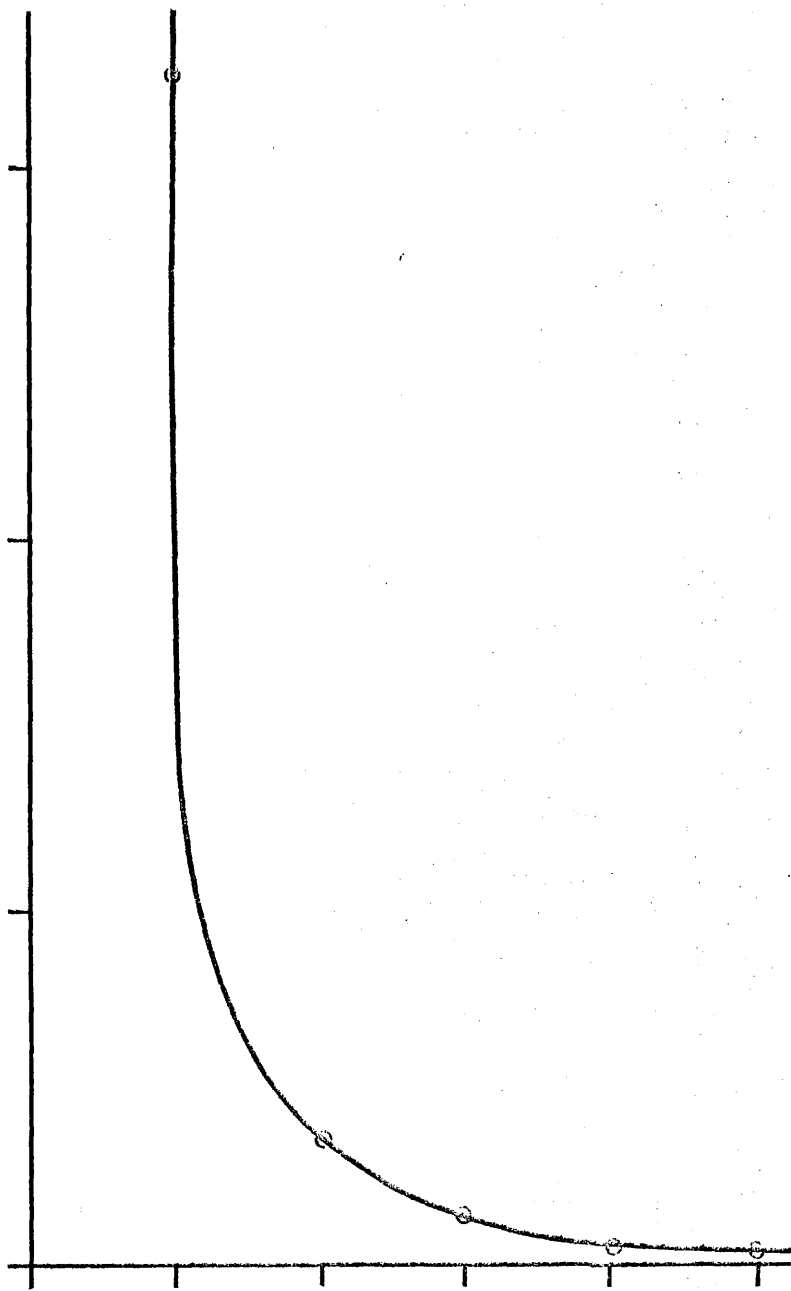
2

3

4

5

Number of washes



amount of radioactivity (cpm ^3H) bound to the protein on the disc was measured in a Packard Tri-Carb Liquid Scintillation Spectrometer (Model 3375). Triplicate discs were processed for each incubated sample. Blanks prepared using colchicine solution only, routinely produced 40 - 80 cpm per disc; heat-denatured protein blanks gave 80 - 160 cpm per disc when tested using soluble or particulate protein preparations. These values were usually ignored, since the levels of radioactivity normally measured in CB-determinations (2000 cpm or higher) greatly exceeded these amounts.

(iii) Usefulness of disc assays compared with other published methods

In a limited number of experiments, CB-activity assayed by the filter disc method (see above) was compared to the activity measured using two other methods described for separating free and bound colchicine.

The first method, of Weisenberg *et al* (1968), involves the use of Sephadex G-100, equilibrated with 10mM P-Mg buffer pH 6.8 in a 1 x 15cm column at 4⁰. Samples of incubation mixture were chilled and applied to the top of the column, and were eluted with ice-cold 10mM P-Mg buffer. The protein, together with the protein-bound colchicine were eluted first, and were separated from the free colchicine as determined by measuring the radioactivity of aliquots of the individual fractions collected. Protein in aliquots of the fractions was determined by the method of Lowry *et al* (see section (h), this chapter). The results are illustrated in Fig. 2.2, which shows the distribution of protein and radioactivity in the effluent. In a parallel experiment, samples of the same incubation mixture were taken for assay by the centrifugation method of Weisenberg (1968). The chilled sample was added to Sephadex DEAE-A50 which had been swollen in distilled water, washed with acid and alkali (as specified by Pharmacia, the makers), and equilibrated in 10mM P-Mg buffer pH 6.8 at 4⁰ in a centrifuge tube, and was allowed to absorb for 5 mins. The tube was then centrifuged and the supernatant discarded. The pelleted Sephadex with absorbed protein and protein-bound colchicine was then washed with five changes of 10mM P-Mg buffer (allowing 5 min/wash) to remove unbound colchicine. The Sephadex and absorbed colchicine were then counted directly in 15ml of Bray's scintillant.

The results obtained using each of these methods are tabulated in Table 2.1. It is apparent from the Table that very similar colchicine binding values for the three methods were obtained, the filter disc method being slightly less quantitative than the other two methods. There are, however, obvious advantages of the filter disc method (many samples can be processed fairly easily at once in a relatively short time; very small samples could be used and a reasonable amount of radioactivity due to bound [^3H] colchicine could be detected) which resulted in this method being adopted for routine use.

Figure 2.2

Distribution of protein and bound colchicine eluted from Sephadex G-100 column

Samples containing protein-bound [^3H]-colchicine and free [^3H]-colchicine were applied to the top of a Sephadex G-100 column equilibrated with 10mM sodium phosphate buffer, pH 6.8, containing 10mM MgCl_2 . 1ml fractions were collected and aliquots were taken for measurement of radioactivity and protein. The first peak of radioactivity obtained is due to protein-bound colchicine; the second peak is due to free colchicine.

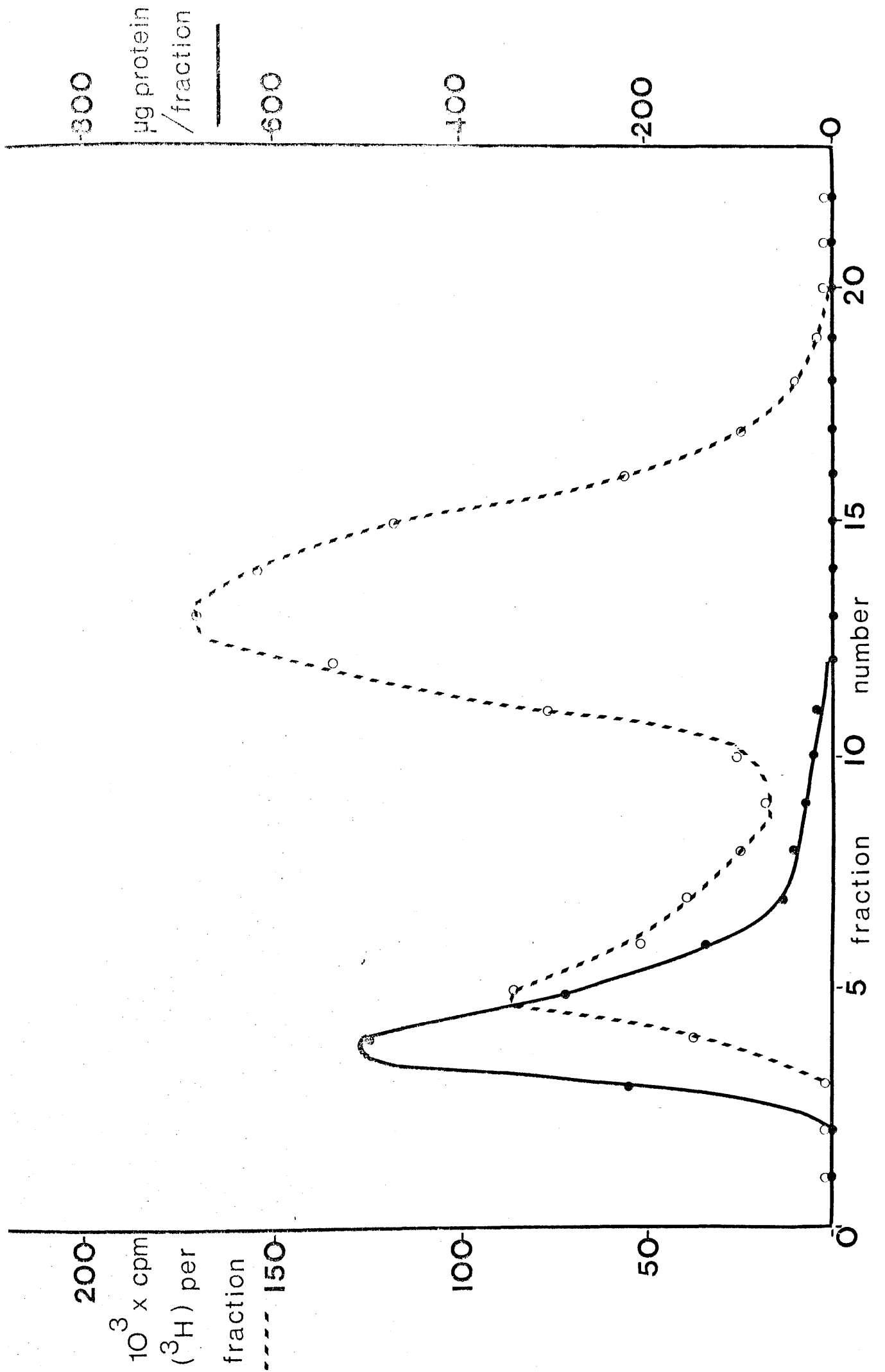


Table 2.1

Comparison of CB-activity determined by different methods

The incubation mixture used in these experiments contained 1.8mg/ml of protein and $2.5 \mu\text{M}$ [^3H]-colchicine in 10mM P-Mg buffer, pH 6.6. Samples were incubated at 37° for 1.5h. 1ml samples were loaded on to the G-100 Sephadex columns; 0.0ml samples were used (per tube) in the centrifugation assay using DEAE-A50 Sephadex; 0.1ml samples (per disc) were used in the DE81 filter disc assays. The results were normalised to correspond to 1ml samples of incubation mixture. The total radioactivity ($[^3\text{H}]$) present in each 1ml of incubation mixture was 1.2×10^6 cpm. Duplicate samples were processed using the column and centrifugation methods, and filter disc assays were carried out in triplicate. It is worthwhile noting that in the centrifugation method the DE-Sephadex, when dissolved in the scintillant, resulted in a quenching of the radioactivity counting by about 40%, found by an internal standardisation method. This has been corrected for in the results shown. In the column and disc methods, only 0.1ml of aqueous sample was added to the scintillant, and this resulted in a quenching of less than 6%.

Table 2.1

Comparison of CB-activity determined by different methods

<u>Method</u>	<u>Sample used</u>	<u>Colchicine-binding (bound cpm x 10³)</u>	<u>Mean % of column value</u>
Sephadex G-100 column chromatography	soluble	256 ± 3	100
Sephadex G-100 column chromatography	particulate	53 ± 1	100
Sephadex DEAE-A50 centrifugation chromatography	soluble	236 ± 13	92
Sephadex DEAE-A50 centrifugation chromatography	particulate	68 ± 2	128
Filter disc	soluble	202 ± 10	80
Filter disc	particulate	48 ± 3	91

(e) Efficiency of counting

The efficiency of counting in the scintillant used was found by measuring the cpm of a specified volume of the stock colchicine solution, of known dpm.

$$\text{From the relation: efficiency} = \frac{\text{cpm} \times 100}{\text{dpm}} \%$$

the efficiency was found to be 48%.

In some of the experiments investigating the kinetics of the binding reaction, the cpm obtained were converted into moles of colchicine bound, on the basis of cpm measured under identical conditions for [³H]-colchicine solutions of known concentration (as described in chapter 5, methods).

(f) Preparation of stock radioactive colchicine solution

Radioactive colchicine obtained had specific radioactivities ranging between 1.7 - 2.2 Ci/mole and contained 250 μ Ci in 0.25ml of a 50% ethanolic solution. This corresponds to solutions of concentration approximately 500 μ M. This was diluted with cold colchicine solution, as shown below, to give a stock solution of final concentration approximately 76 μ M:

	<u>Volume used</u>	<u>Final conc.</u>
original radioactive colchicine solution (500 μ M)	0.25ml	20 μ M
unlabelled colchicine solution (1400 μ M)	0.25ml	56 μ M
water	5.75ml	
	<u>6.25ml</u>	<u>76 μM</u>

The specific radioactivity of the stock colchicine solution was 33,000 cpm/ μ l (at 48% efficiency this corresponds to 530 cpm/pmole). When diluted 30 times in the incubation mixtures (20 μ l in 0.6ml), the final colchicine concentration obtained was 2.5 μ M, and this was routinely used in the standard assay.

(g) Preparation of scintillation fluid (Bray's scintillant)

It was convenient to use a scintillant which was miscible with water, in which the efficiency of counting was not too low. Bray's (1960) scintillant was tried, and compared to a toluene-based scintillant gave favourable results on both points. Bray's scintillation mixture is a dioxan-based fluid, and is capable of accommodating up to about 20% of its own volume of water while giving reasonable counting efficiencies.

This water will cause quenching in the sample (it will interfere with the production of photons in the scintillator) and a lower cpm will be recorded than that which is actually present. For this reason a fairly large amount of naphthalene was added to the scintillant, to increase the efficiency of the water-quenched system.

Most scintillation counters operate at a reduced temperature (around 4°C) in order to minimise background 'noise', since electron mobility decreases as the temperature is reduced. As dioxan freezes at 8°C, an amount of ethylene glycol was incorporated into the scintillant to overcome this difficulty. Another addition was ethoxyethanol, (Cellosolve), which was used to overcome other problems concerned with the size of the aqueous sample, adsorption of salts onto the wall of the vial, and precipitation of the sample material. The remaining constituents were 2,5-diphenyloxazole (PPO) and 1,4-di-(2-(5-phenyloxazolyl))-benzene (POPOP). These 'fluors' in the scintillant convert the nuclear radiation produced by the sample into light energy, or photons, which are detected and amplified by the scintillation counter. Therefore, a scintillation fluid was prepared in which the following chemicals were dissolved, and made up to 1 litre, with scintillation grade dioxan:

150g naphthalene
8g PPO
0.6g POPOP
100ml ethoxyethanol
20ml ethylene glycol

(See Proceedings of the Beckman Summer School, 1967)

This scintillant differs from that used by Bray in that methanol was replaced by ethoxyethanol, and the naphthalene, PPO and POPOP amounts were increased for greater counting efficiency. General Purpose grade naphthalene was used, which was reported to give almost the same efficiency as scintillation grade, and was very much cheaper.

(h) Estimation of protein by the method of Lowry et al (1951)

The main reason for estimating brain protein by the method of Lowry is that lipids, which are present to a significant extent in brain tissue, do not appear to interfere significantly with the colour reaction produced. Up to 6% only of the colour produced by whole tissue is attributable to lipid interference, whereas a N-based determination (Kjeldahl) could be up to 20% in error. Also, the Lowry method is

sensitive, detecting even a few μg of protein, in the procedure described below. To increase the sensitivity of the method, the volumes of all the reagents used were decreased by the same proportion, to yield a more concentrated colour for a given amount of protein.

The following reagent solutions were used:

- (A) 2% Na_2CO_3 in 0.1N NaOH
- (B) 0.5% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 1% sodium citrate (citrate here replaces tartrate, producing a more stable reagent)
- (C) 50 parts of (A) freshly mixed with 1 part of (B)
- (D) Folin-Ciocalteu reagent which was generally diluted 1:2.3 with water to give a solution which was 1N with respect to acid.

Serum albumen 100 $\mu\text{g}/\text{ml}$ (protein standard)

Assays were carried out as follows: samples or standards containing up to 100 μg of protein in a total volume of 0.8ml were mixed with 4ml of reagent (C) and then allowed to stand for 10 min. at room temperature. 0.4ml of reagent (D) was then pipetted rapidly into the mixture with thorough mixing and the blue colour produced after standing for at least 20 min. was read in a Beckman spectrophotometer at 700 nm against a reagent blank. Standard curves were obtained for each determination (see Fig. 2.3) and were used to estimate the amount of protein in the samples. Corrections were made for interfering substances (e.g. high salt concentrations or Vinca alkaloids) where necessary.

(i) Thin-layer chromatography of colchicine

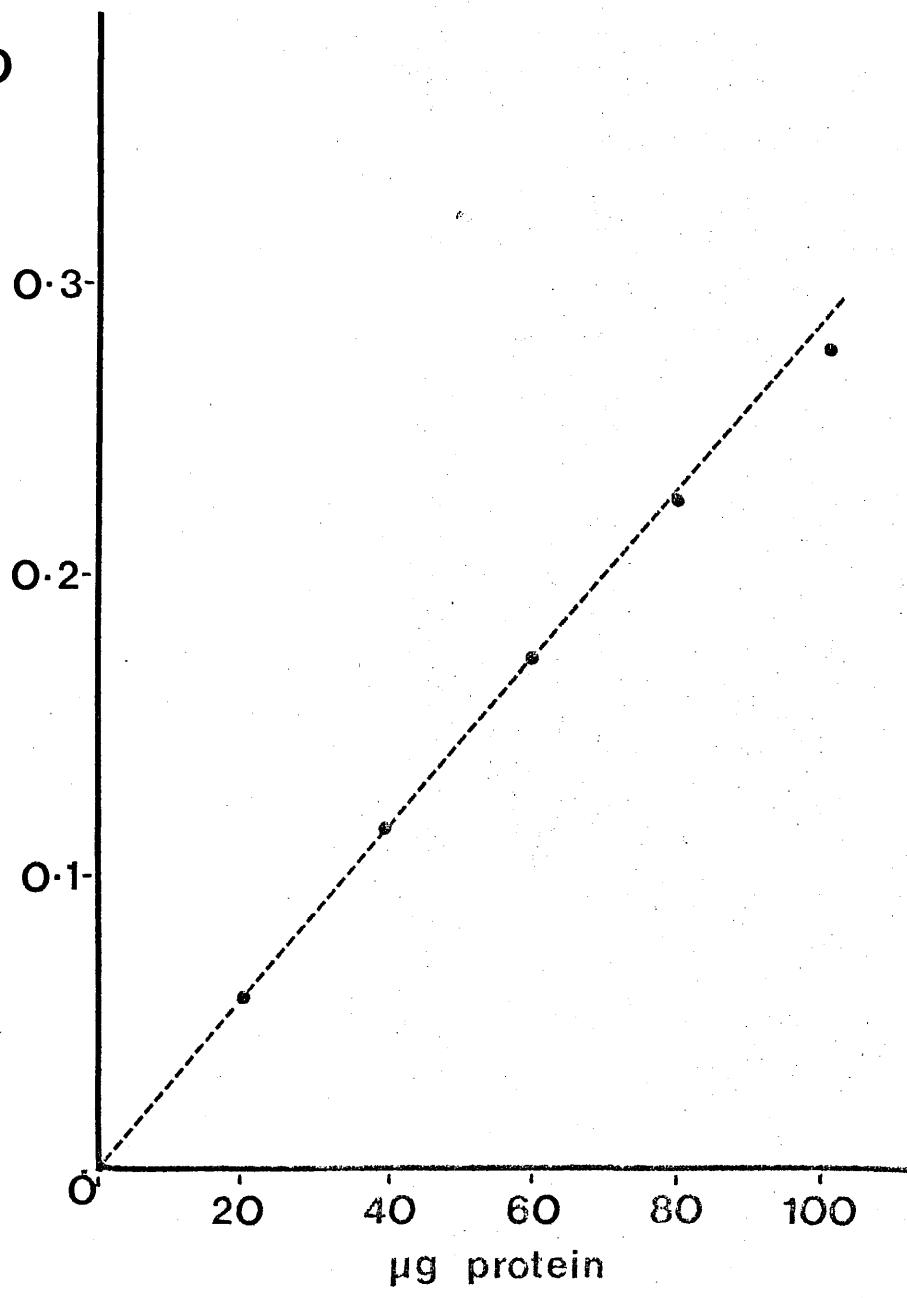
To test the purity of the [^3H]-colchicine used in this work, samples were analysed by thin-layer chromatography, as described by Wilson and Friedkin (1966). Glass plates 20cm x 20cm were coated with a layer of Silica Gel G (Kieselgel G, Merck), 250 μ thick, by applying a slurry of 25g/50ml water. Plates were dried in a 100 $^\circ$ oven for 1h. Up to 50 μg of colchicine were applied per spot, and the chromatograms were developed with methanol until the front had almost reached the top of the plate. (This took about 1h.) The plates were allowed to dry in air, then were viewed under UV light to locate the positions of the colchicine and of any impurities or breakdown products which might have been present. In fact, only one spot could be detected, approximately half-way up the plate, giving an R_f value of 0.56 ± 0.02 . This corresponds closely to the R_f value found by Wilson (1966) using identical chromatographic procedures. This was clearly distinguished from the R_f for deacetylcolchicine (0.4) & isodeacetylcolchicine (0.3).

Figure 2.3

Estimation of protein by the method of Lowry et al
(1951)

This reference graph was obtained using known amounts of bovine serum albumen. For method, see text. Each sample was read against a reagent blank which contained no protein.

E_{700}



To check the radiochemical purity of [^3H]-colchicine, samples of the labelled material (about 5 nmoles, $\approx 10^7$ cpm) were chromatographed as described above. Samples of silica gel were then scraped off the plate at 1cm intervals along its length, and were counted in 5ml of Bray's scintillant. Only 1 peak of radioactivity was obtained, showing that the [^3H]-colchicine was radiochemically pure. This was confirmed by radioautography when the dried plate was left in contact with an X-ray plate for 1 month, again indicating that there was no significant radiochemical impurity present.

Similarly, the nature of the protein-bound radioactivity was investigated after incubation of protein samples with [^3H]-colchicine. Soluble and particulate proteins were incubated with 2.5 μM [^3H]-colchicine by the standard procedure, and 0.1ml samples were applied to DE81 filter discs as previously described (section (d)). After washing to remove unbound [^3H]-colchicine, the bound radioactivity from 5 discs was eluted with a total of 2ml methanol. A total of 0.2ml of each sample was then loaded per spot (this corresponds to about 6,000 cpm/spot in the case of the soluble preparation, and about 1,000 cpm in the case of the particulate preparation, calculated from the cpm adsorbed onto DE81 filter discs from an aliquot of the incubation mixture.) 50 μg samples of the standards were included and the plate was developed as previously described. Silica gel scrapings taken at 1cm intervals along the length of the plate showed that there was 1 spot in the position of the unchanged colchicine. This was confirmed by radioautography of the chromatographic plate using X-ray film and left in contact for 3 months, indicating that the radioactivity bound to the protein is, in fact, due to colchicine bound, and not any other derivative.

(j) Polyacrylamide gel electrophoresis

This technique was used to check the purity of purified microtubular protein, and to partly identify microtubular protein in crude mixtures. Two systems were used which both included the detergent sodium dodecyl (lauryl) sulphate (SDS) in which the proteins were separated mainly according to size rather than charge. In most experiments, 10% gels were used. Acrylamide was obtained from Serva, Heidelberg, and was of the highest grade of purity. It was stored at 4 $^{\circ}\text{C}$.

The first method used was mainly as described by Weber and Osborn (1969) who used a continuous phosphate buffer system at pH 7.0. The following solutions were prepared:

Acrylamide

10.5g acrylamide + 0.15g NN'-methylenebisacrylamide, made up to 50ml with water (freshly prepared).

Gel buffer

21.5g anhydrous Na_2HPO_4 + 8.5g NaH_2PO_4 + 2g SDS, made up to 1 litre with water (this gives approximately 0.2M sodium phosphate buffer pH 7.0, containing 0.2% SDS).

Incubation buffer

2.15g anhydrous Na_2HPO_4 + 0.85g NaH_2PO_4 + 2g SDS + 2ml B-mercapto-ethanol, made up to 100ml with water (this gives approximately 0.2M sodium phosphate buffer pH 7.0 containing 2% SDS and 2% B-mercapto-ethanol).

Ammonium persulphate

0.15g in 10ml water, freshly prepared.

Gel stain

1.25g Coomassie brilliant blue dissolved in 454ml 50% methanol followed by addition of 46ml glacial acetic acid, and filtration.

(i) Preparation of gels

Gels were prepared using the solutions described above, as follows: 20ml gel buffer and 18ml acrylamide solution were separately de-aerated using a suction pump for a few minutes, and were then mixed. 2ml ammonium persulphate solution was added together with 0.06ml NNN'-N'-tetramethylethylenediamine (TEMED); these chemically polymerise the acrylamide at room temperature into a gel in about 15 min. The gel solution was transferred to siliconised glass tubes of inside diameter 5mm, length 8-10cm, which were closed by plastic caps at one end, filling the tubes to within 1cm of the top. When gelation had finished and the gels were to be used, the plastic caps were removed and the tubes were placed in the electrophoresis apparatus.

(ii) Preparation of protein samples

Protein samples were prepared for electrophoresis as follows: protein solutions containing approximately 2mg protein/ml were diluted with an equal volume of incubation buffer, and were incubated at 37° for at least an hour, or at 50° for about 20 min. To 0.1ml of this solution was then added 1 drop of glycerol (to increase the density of the sample) and 1 drop of a saturated aqueous solution of bromophenol blue (tracking dye).

(iii) Electrophoresis

The prepared protein sample was applied to the top of the gel and was electrophoresed (the anode being in the top of the apparatus) using gel buffer diluted with an equal volume of water as the electrophoresis buffer. Using a current of 6mA/gel (maximum) electrophoresis

took about 6h. to be completed; alternatively, a lower current was frequently used (2mA/gel) when the electrophoresis was completed in 18-20h.

(iv) Fixing and staining

The gels were removed from the glass tubes and their length and the distance the tracker dye had migrated from the origin were measured. The gels were placed in 10% sulphosalicylic acid (or 10% TCA) at room temperature for at least 30 min. to precipitate the protein in the gel. The gels were then overstained with Coomassie brilliant blue for about 1h. , followed by diffusion destaining in 7% acetic acid containing 50ml methanol/litre.

(v) Estimation of molecular weights

Proteins of known molecular weight were electrophoresed in each experiment, and their mobilities were calculated from the relation

$$\text{mobility} = \frac{\text{length before destaining}}{\text{length after destaining}} \times \frac{\text{distance ptn migrated}}{\text{distance dye migrated}}$$

When these mobilities were plotted against the logarithm of the molecular weight, a standard curve was obtained (see Figs. 2.4 & 2.5) which was used to estimate the molecular weights of unknown proteins from their calculated mobilities.

It was discovered that tubulin (CB-protein) could be split into two bands of equal size if a discontinuous buffer system was used and urea was incorporated (similar to that described by Laemmli, 1970). This method involved the following solutions:

Gel buffer

0.75M tris-HCl buffer, pH 8.8, containing 0.2% SDS and 10M urea.

Incubation buffer

0.125M tris-HCl buffer, pH 6.8, containing 4% SDS, 10% B-mercapto-ethanol and 10M urea.

Electrophoresis buffer

3.025g tris + 14.4g glycine + 1g SDS, made up to 1 litre with water (this gives a solution containing 0.025M tris-0.192M glycine, with a final pH of 8.3, containing 0.1% SDS).

Gels were prepared (using half the persulphate and TEMED previously stated) and the protein samples were incubated as described above. Electrophoresis was performed using undiluted electrophoresis buffer, and a maximum current of 2.5mA/gel; electrophoresis was completed in about 2h. Gels were then processed exactly as described above. For results, see Fig. 2.6.

Figure 2.4

Estimation of molecular weights by SDS-polyacrylamide gel electrophoresis

This reference graph was obtained using proteins of known molecular weight which were electrophoresed in 0.1M sodium phosphate buffer, pH 7.0, containing 0.1% SDS as described in the text. Mobilities were calculated from the distance moved through the gel by the protein as described in the text.

M.Wt. $\times 10^3$

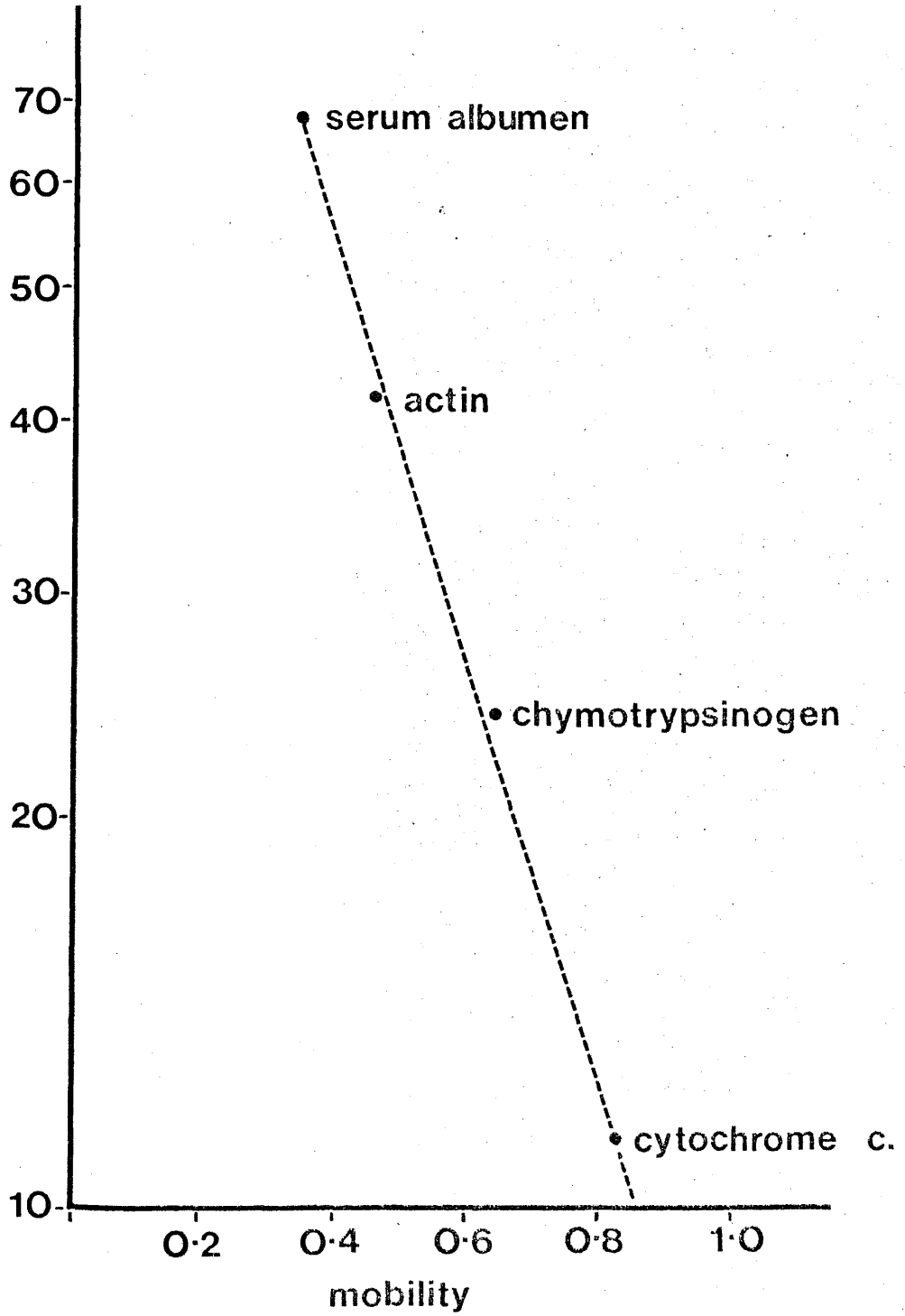


Figure 2.5

SDS-polyacrylamide gel electrophoresis of reference proteins

Gels 1 - 3 were loaded with approximately 50 μg of each protein and were electrophoresed in the phosphate-SDS buffer system of Weber and Osborn, as described in the text. Gels were fixed in 10% sulphosalicylic acid, overstained with Coomassie brilliant blue, then diffusion destained. Gels 4 - 7 were loaded with approximately 50 μg of each protein and were electrophoresed in tris buffer containing SDS and urea (see text). The gels were fixed and stained as above.

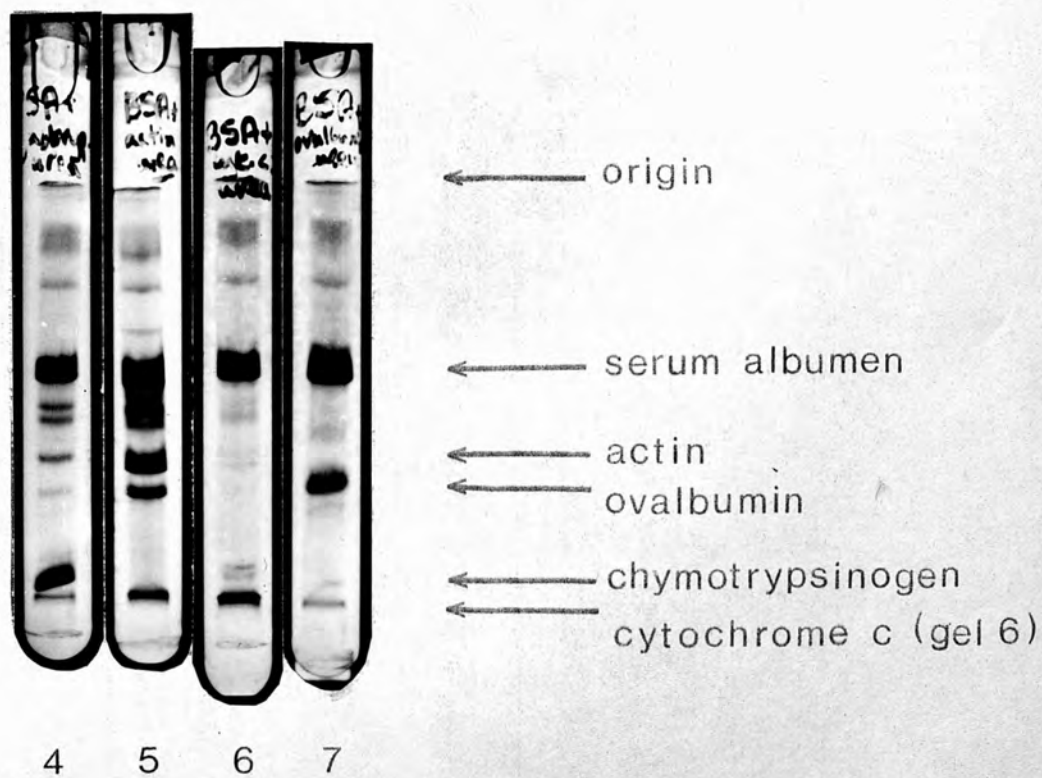
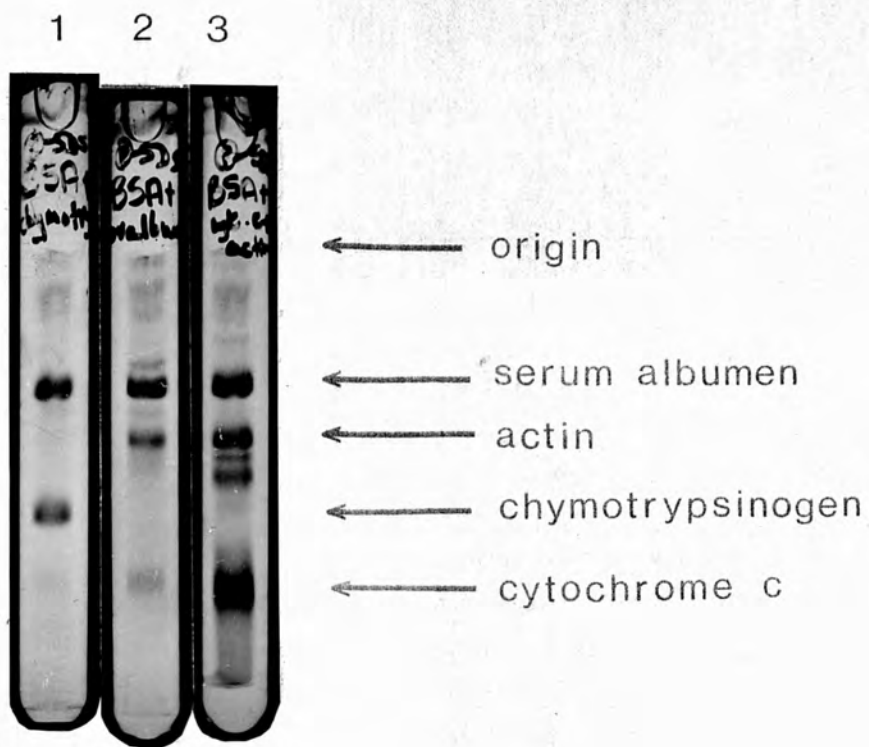
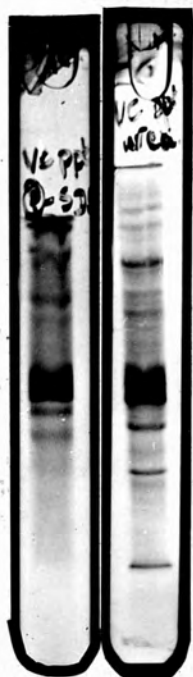


Figure 2.6

SDS-polyacrylamide gel electrophoresis of rat brain tubulin

Gel 1 was loaded with approximately 100 μg of a partly purified tubulin preparation and was electrophoresed in phosphate-SDS buffer (see text for details). Gel 2 was similarly treated, and electrophoresed in tris buffer containing SDS and urea (see text for details). Gels were fixed, then stained with Coomassie brilliant blue. Gel 1 reveals one main protein band, corresponding to a molecular weight of approximately 58,000 while in Gel 2 two main bands are apparent, corresponding to molecular weights of 58,000 and 56,000.



molecular weight

58,000
56,000

1 2

(k) Radioautography of polyacrylamide gels

This technique was used when the distribution of protein-bound radioactivity needed to be found (see Chapter 6, Results, Section (d) and Fig. 6.5). The proteins in the samples and their bound radioactivity were separated by polyacrylamide gel electrophoresis as described in Section (j) of this Chapter. The stained gels were then sliced longitudinally using the apparatus of Fairbanks et al (1965). The gel was clamped in a perspex holder, a cutter was inserted at the top and was drawn evenly down the apparatus until the whole length of the gel had been sliced. Two inner flat slices (approximately 1mm thick) were obtained, and two outer, rounded slices. The inner slices were then placed closely together on moistened, high-wet strength, filter paper which was placed on a perspex slab from which channels had been cut for drainage as the slices were dried. The apparatus was covered with polythene to make it air tight, and was evacuated overnight using a suction pump. It was found that the gel slices dried more evenly if an infra-red lamp was placed about 18" above the apparatus so that a temperature of about 50^o was maintained on the surface of the drier for the first few hours. The dried slices, adhering to the filter paper, were then left in contact with X-ray film to obtain a radioautograph of the separation on the gel.

CHAPTER THREE

Investigation of the distribution of brain
CB-protein from different species, neuro-
anatomical locations and during development.

Introduction

Most of the CB-activity present in homogenates of various types of non-neural cells (Borisy and Taylor 1967; Wilson and Friedkin 1968; Wilson 1970) appears to be recovered in the high-speed soluble fraction. The CB-activity present in the high-speed soluble extracts of homogenates was therefore assumed, at that time, to be a reasonable measure of total binding activity of the various tissues examined, including mammalian brain.

However, preliminary data by Dutton and Barondes (1969) indicated that about half the total CB-activity of mouse brain homogenates appeared to be firmly bound to the crude particulate fraction obtained after high-speed centrifugation. It was therefore of interest to investigate the extent to which CB-activity in brain was associated with the particulate fraction, and in this Chapter, results of studies on the distribution of CB-activity in crude soluble and particulate fractions in rat, rabbit and guinea-pig brain will be summarised. In addition, a study was made of the distribution of CB-activity in various gross anatomical regions of adult brain. Finally, a limited number of experiments were carried out to compare the distribution of CB-activity in immature and adult rat and rabbit brain.

Materials and Methods

The brains from adult rats and guinea-pigs were obtained as described in Chapter 2 (b). Young rats (1-2 days old, all from the same litter) were killed directly by decapitation, and the brains removed. Adult rabbit brain was obtained from animals which had been injected with Nembutal in the spinal cord and in the marginal ear vein, then killed by air injection and decapitated. 28-day rabbit foetuses were removed from the anaesthetised mother and were killed by decapitation, and the brains removed. All the tissues were chilled on ice as quickly as possible after removal, and transferred to filter paper moistened with cold homogenising medium. Dissection of the adult brains into various gross anatomical regions was performed as described by Dahl et al (1970).

Tissues were homogenised and centrifuged, and the crude soluble and particulate extracts obtained as described in Chapter 2 (b). CB-activity was measured in the extracts by the DE filter disc assay described in Chapter 2 (d). The homogenising medium used was 0.32M sucrose containing 10mM sodium phosphate buffer, pH 6.8, and 10mM $MgCl_2$. These solutions were prepared as described in Chapter 2 (c).

Results are expressed as specific activities (SA's), which are cpm of colchicine bound per mg protein; and as relative specific activities

(RSA's), which are obtained from the relation $\frac{\% \text{ recovered bound cpm}}{\% \text{ recovered protein}}$.

The latter facilitates comparison of CB-activities in the various sub-fractions with that of the parent fraction, whose RSA is taken to be 1.

Results

(a) Distribution of CB-activity in adult rat, rabbit and guinea-pig brain

Preliminary experiments with rat brain indicated that about half the total homogenate CB-activity was associated with the particulate fraction. Further investigations, using also rabbit and guinea-pig brain, confirmed this finding. The total amount of protein and CB-activity recovered in extracts were very similar in the different species studied when expressed on a wet tissue weight basis (see Fig. 3.1). The distribution of protein and CB-activity between the crude soluble and particulate extracts was also similar, approximately 20% of the protein and 50% of the CB-activity being recovered in the soluble fraction, the remainder in the pelleted material (see Table 3.1). Binding of colchicine in the particulate fraction of mammalian brain therefore appeared important, accounting for half that found in brain homogenates.

(b) Changes in distribution of CB-activity during development of rat and rabbit brain

Preliminary experiments showed that the CB-activity present in soluble extracts of immature brain was higher than that for adult brain. The distribution of CB-activity during development of rat and rabbit brain was therefore investigated further.

It was found, for both species, that the total amount of protein increased during development, but the total recovered CB-activity decreased when expressed on a wet tissue weight basis (see Fig. 3.2). The distribution of protein also changed during development; the proportion of soluble protein decreased, accompanied by an increase in particulate protein, for both species. The distribution of CB-activity was also found to alter during development of the rat brain - the proportion of soluble CB-activity decreasing, and particulate CB-activity increasing. However, no significant change in distribution of CB-activity was found during development of rabbit brain (see Table 3.2).

Figure 3.1

Protein content and CB-activity in soluble and particulate extracts of mammalian brain

Soluble (S) and particulate (P) extracts of brain were prepared as described in Chapter 2. Samples were removed for determination of protein and CB-activity (see Chapter 2). Data refer to results obtained from 1g wet weight of tissue and are the means of 6 separate experiments (rat), 3 separate experiments (rabbit), and 2 separate experiments (guinea-pig). Actual total brain weights were: rat 1.7g; rabbit 10g; guinea-pig 3.2g. See also Table 3.1.

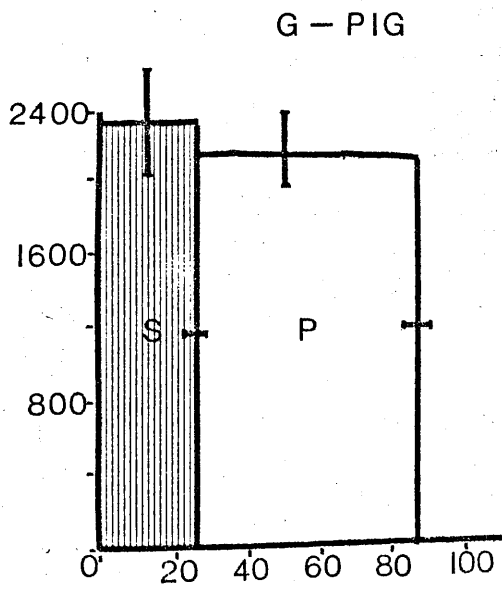
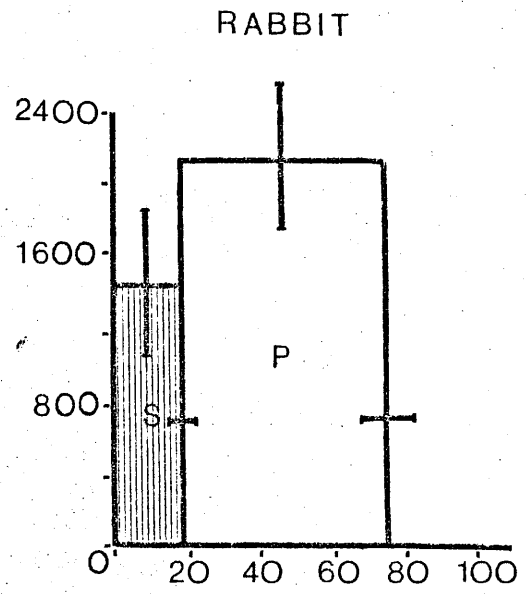
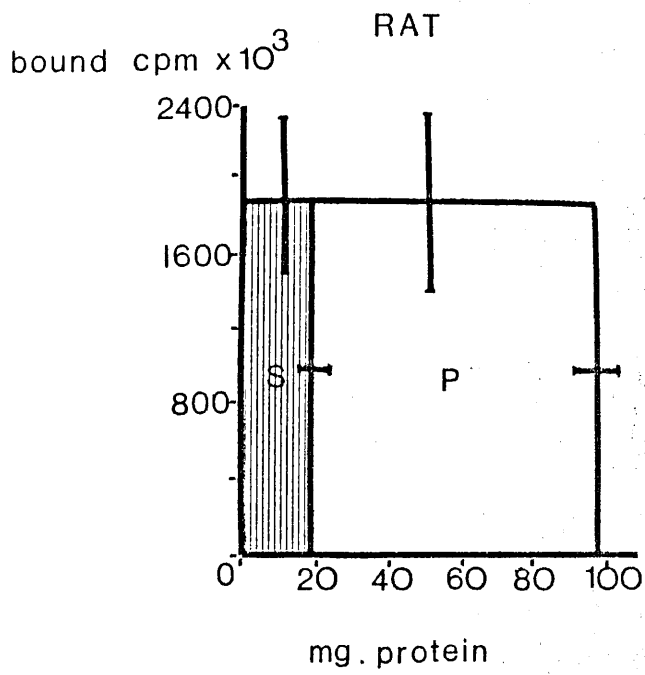


Table 3.1

Distribution of protein and CB-activity in soluble
and particulate extracts of mammalian brain

Soluble and particulate extracts of brain were prepared as described in Chapter 2. Aliquots were removed for determination of protein and CB-activity (as described in Chapter 2). Results are the means of 6 separate experiments (rat), 3 separate experiments (rabbit) and 2 separate experiments (guinea-pig).

See also Figure 3.1.

Table 3.1

Distribution of protein and CB-activity in soluble and particulate extracts of mammalian brain

	<u>% recovered protein</u>	<u>% recovered CB-activity</u>	<u>R.S.A.</u>	<u>specific activity</u> ($\times 10^3$)
<u>Rat</u>				
soluble	20 \pm 3	48 \pm 6	2.4 \pm 0.4	95 \pm 20
particulate	80 \pm 4	52 \pm 7	0.65 \pm 0.1	25 \pm 4
<u>Rabbit</u>				
soluble	24 \pm 3	47 \pm 5	2.0 \pm 0.3	93 \pm 30
particulate	76 \pm 2	53 \pm 5	0.7 \pm 0.1	39 \pm 8
<u>Guinea-pig</u>				
soluble	30 \pm 3	52 \pm 2	1.8 \pm 0.2	93 \pm 20
particulate	70 \pm 3	48 \pm 2	0.7 \pm 0.1	36 \pm 5

Figure 3.2

Protein content and CB-activity in soluble and particulate extracts of immature and adult brain

Soluble (S) and particulate (P) extracts of brain were prepared as described in Chapter 2. Samples were removed for determination of protein and CB-activity (see Chapter 2). Data presented refer to results obtained from 1g wet weight of tissue, and are the means of 3 separate experiments (foetal rabbit, adult rabbit and 1-day old rat) and of 6 separate experiments (adult rat).

Actual total brain weights were: foetal rabbit 1g; adult rabbit 10g; 1-day old rat 0.3g; adult rat 1.7g. See also Table 3.2.

bound cpm x10³

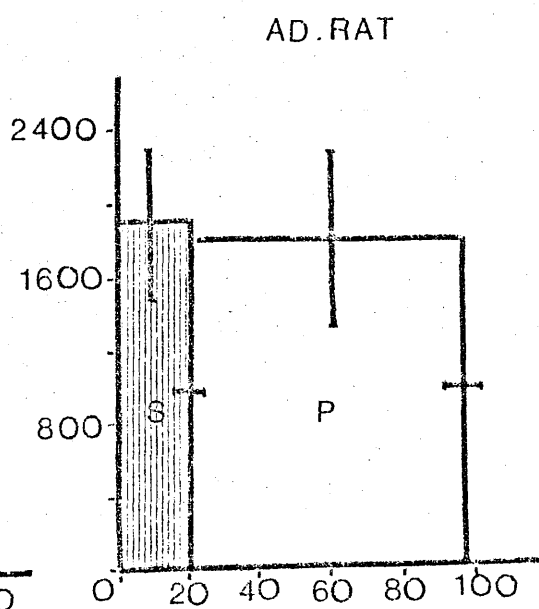
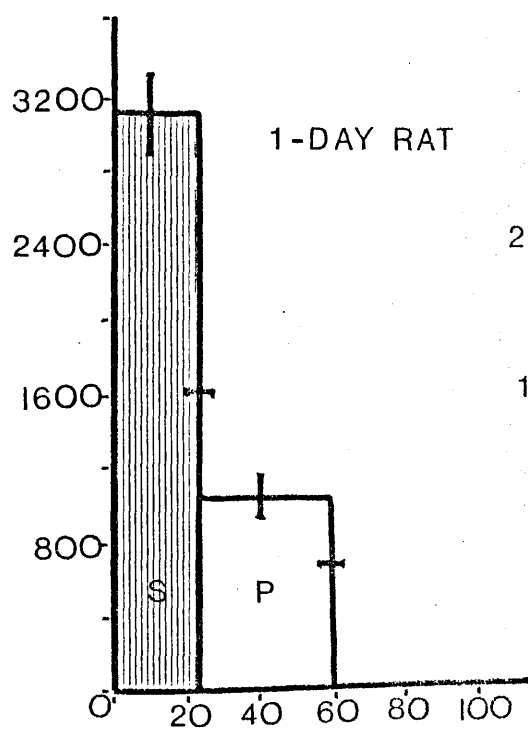
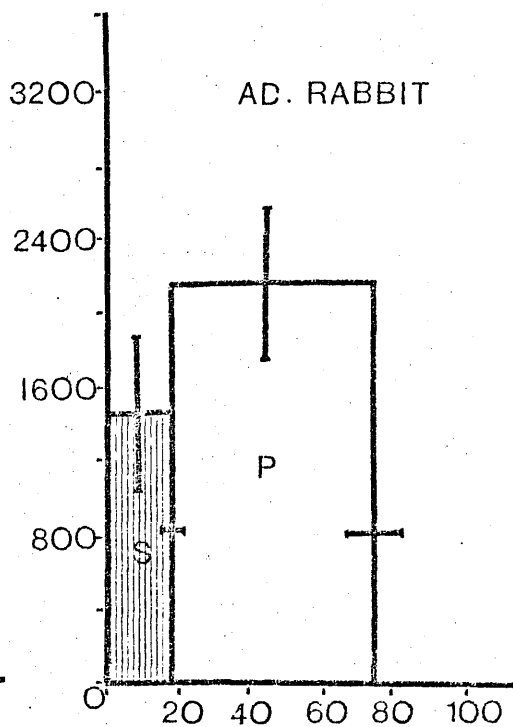
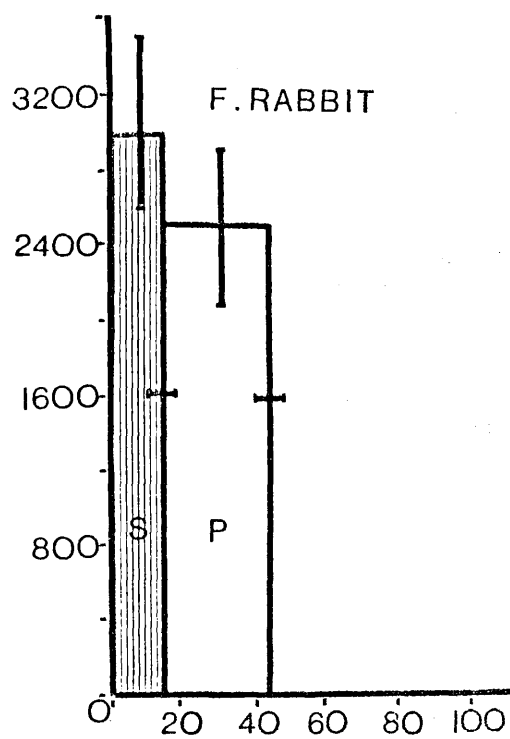


Table 3.2

Distribution of protein and CB-activity in soluble
and particulate extracts of immature and adult brain

Soluble and particulate extracts of brain were prepared as described in Chapter 2. Aliquots were removed for determination of protein and CB-activity (as described in Chapter 2). Results are the means of 3 separate experiments (foetal rabbit, adult rabbit, 1-day old rat) and 6 separate experiments (adult rat).

See also Figure 3.2.

Table 3.2

Distribution of protein and CB-activity in soluble and particulate extracts of immature and adult brain

	<u>% recovered protein</u>	<u>% recovered CB-activity</u>	<u>R.S.A.</u>	<u>specific activity</u> ($\times 10^3$)
<u>Foetal rabbit</u>				
soluble	34 \pm 4	54 \pm 6	1.6 \pm 0.3	205 \pm 40
particulate	66 \pm 4	46 \pm 6	0.7 \pm 0.1	90 \pm 20
<u>Adult rabbit</u>				
soluble	24 \pm 3	47 \pm 5	2.0 \pm 0.3	93 \pm 30
particulate	76 \pm 2	53 \pm 5	0.7 \pm 0.1	39 \pm 8
<u>1-day old rat</u>				
soluble	36 \pm 2	72 \pm 4	2.0 \pm 0.3	143 \pm 30
particulate	64 \pm 3	28 \pm 4	0.45 \pm 0.1	26 \pm 4
<u>Adult rat</u>				
soluble	20 \pm 3	48 \pm 6	2.4 \pm 0.4	95 \pm 20
particulate	80 \pm 4	52 \pm 7	0.65 \pm 0.1	25 \pm 4

(c) Distribution of CB-activity in various gross anatomical regions of brain

Different anatomical regions of the brain have assigned to them different functions, and the morphology of the tissue from different areas also varies. Therefore, soluble and particulate colchicine-binding in different gross anatomical areas of brain was studied in three different species.

The total amount of protein in homogenates of the different regions and its distribution between the soluble and particulate fractions were very similar within each of the species studied (see Table 3.3). However, the total CB-activity in homogenates of the different regions of adult brain differed (see Fig. 3.3); the CB-activity in the cerebellum and brain stem areas was significantly lower than that measured in the thalamus/hypothalamus and cortex regions, when expressed on a wet tissue weight basis. These results confirm those of Dahl *et al* (1970) for rat and rabbit brain. Also, CB-activity in homogenates of the different regions of foetal rabbit brain were all found to be significantly higher than those measured in the adult animal (this also confirms the observations made in Section (b) of this Chapter).

The distribution of CB-activity between the soluble and particulate fractions obtained from homogenates of the different regions of adult brain also differed (see Table 3.4) notably in that a low value for the brain stem soluble CB-activity was obtained compared to that measured in the soluble fractions from the other areas of each species studied.

Specific activities of the fractions from each of the regions are compared in Fig. 3.4 from which it is apparent that the pattern of CB-activity found in the homogenates of these samples is reflected in that recovered in the soluble preparations, i.e. for each of the adult species studied the brain stem soluble fraction had a significantly lower CB-activity than that measured in the soluble fractions from other regions of the brain. However, the soluble fractions obtained from the various regions of foetal rabbit brain showed no significant difference in CB-activity. In addition, it was found that CB-activity in the soluble and particulate fractions from immature brain was significantly higher than that in the corresponding fraction from adult brain.

The specific activities of the particulate fractions were not significantly different within each of the adult species studied. However, in the immature animal, the brain stem particulate preparation had a much lower CB-activity than that measured in particulate preparations from the other areas.

Table 3.3

Protein content and distribution in various
anatomical locations of brain

Soluble and particulate extracts of brain (as indicated) were prepared as described in Chapter 2. Aliquots were removed for determination of protein (see Chapter 2). The results refer to protein obtained from 1g wet weight of each tissue, and are the means of 4 separate experiments (rat) and 2 separate experiments (guinea-pig, rabbit and foetal rabbit). Actual total wet weights of the tissues are given in the legend to Figure 3.3.

See also Figure 3.4, Figure 3.5 and Table 3.4.

Table 3.3

Protein content and distribution in various anatomical locations of brain

	<u>mg protein</u>	<u>% recovered protein</u>
<u>Rat</u>		
Cortex:		
soluble	22.1 ± 2	21 ± 2
particulate	82.2 ± 5	79 ± 2
	<u>104.3</u>	
Thalamus:		
soluble	20.7 ± 3	21 ± 2
particulate	79.5 ± 4	79 ± 4
	<u>100.2</u>	
Cerebellum:		
soluble	18.8 ± 2	23 ± 2
particulate	64.2 ± 5	77 ± 3
	<u>83.0</u>	
Brain stem:		
soluble	13.8 ± 1	17 ± 1
particulate	67.8 ± 3	83 ± 2
	<u>81.6</u>	
<u>Guinea-pig</u>		
Cortex:		
soluble	15.7 ± 3	20 ± 1
particulate	62.6 ± 10	80 ± 1
	<u>78.3</u>	
Thalamus:		
soluble	14.6 ± 2	19 ± 1
particulate	63.3 ± 10	81 ± 1
	<u>77.9</u>	
Cerebellum:		
soluble	17.6 ± 2	21 ± 1
particulate	66.3 ± 10	79 ± 2
	<u>83.9</u>	
Brain stem:		
soluble	13.5 ± 1	18 ± 1
particulate	63.0 ± 8	82 ± 1
	<u>76.5</u>	

Continued

Table 3.3

(Continued)

Protein content and distribution in various
anatomical locations of brain

	<u>mg protein</u>	<u>% recovered protein</u>
<u>Rabbit</u>		
Cortex:		
soluble	13.7 ± 1	20 ± 2
particulate	<u>55.7 ± 7</u>	80 ± 2
	<u>69.4</u>	
Thalamus:		
soluble	13.9 ± 1	19 ± 2
particulate	<u>58.9 ± 10</u>	81 ± 2
	<u>72.8</u>	
Cerebellum:		
soluble	15.7 ± 1	23 ± 3
particulate	<u>54.7 ± 8</u>	77 ± 3
	<u>70.4</u>	
Brain stem:		
soluble	10.7 ± 1	17 ± 3
particulate	<u>53.0 ± 13</u>	83 ± 3
	<u>63.7</u>	
<u>Foetal rabbit</u>		
Cortex:		
soluble	14.5 ± 1	31 ± 4
particulate	<u>33.3 ± 4</u>	69 ± 4
	<u>47.8</u>	
Thalamus:		
soluble	13.0 ± 1	29 ± 3
particulate	<u>33.1 ± 6</u>	71 ± 3
	<u>46.1</u>	
Cerebellum:		
soluble	12.7 ± 1	30 ± 4
particulate	<u>31.2 ± 6</u>	70 ± 4
	<u>43.9</u>	
Brain stem:		
soluble	12.5 ± 1	28 ± 3
particulate	<u>32.5 ± 7</u>	72 ± 3
	<u>45.0</u>	

Figure 3.3

CB-activity in homogenates of different regions of mammalian brain

10% homogenates of cerebral hemisphere (cortex) (C), thalamus/hypothalamus region (T), cerebellum (Ce) and brain stem (S) from brains of each of the species indicated were made in 0.32M sucrose containing 10mM phosphate buffer, pH 6.8, and 10mM MgCl₂. CB-activity was determined in samples of each as described in Chapter 2. Results given refer to CB-activity measured from 1g wet weight of each tissue, and are the means of 4 separate experiments (rat), and 2 separate experiments (guinea-pig, adult rabbit and foetal rabbit). Actual total wet weights of the different tissues were:

Rat

C = 1g T = 0.3g Ce = 0.24g S = 0.14g

Guinea-pig

C = 2g T = 0.5g Ce = 0.5g S = 0.2g

Adult rabbit

C = 5g T = 1.5g Ce = 1.5g S = 1g

Foetal rabbit

C = 0.5g T = 0.1g Ce = 0.2g S = 0.1g

See also Table 3.3, Table 3.4, Figure 3.4 and Figure 3.5.

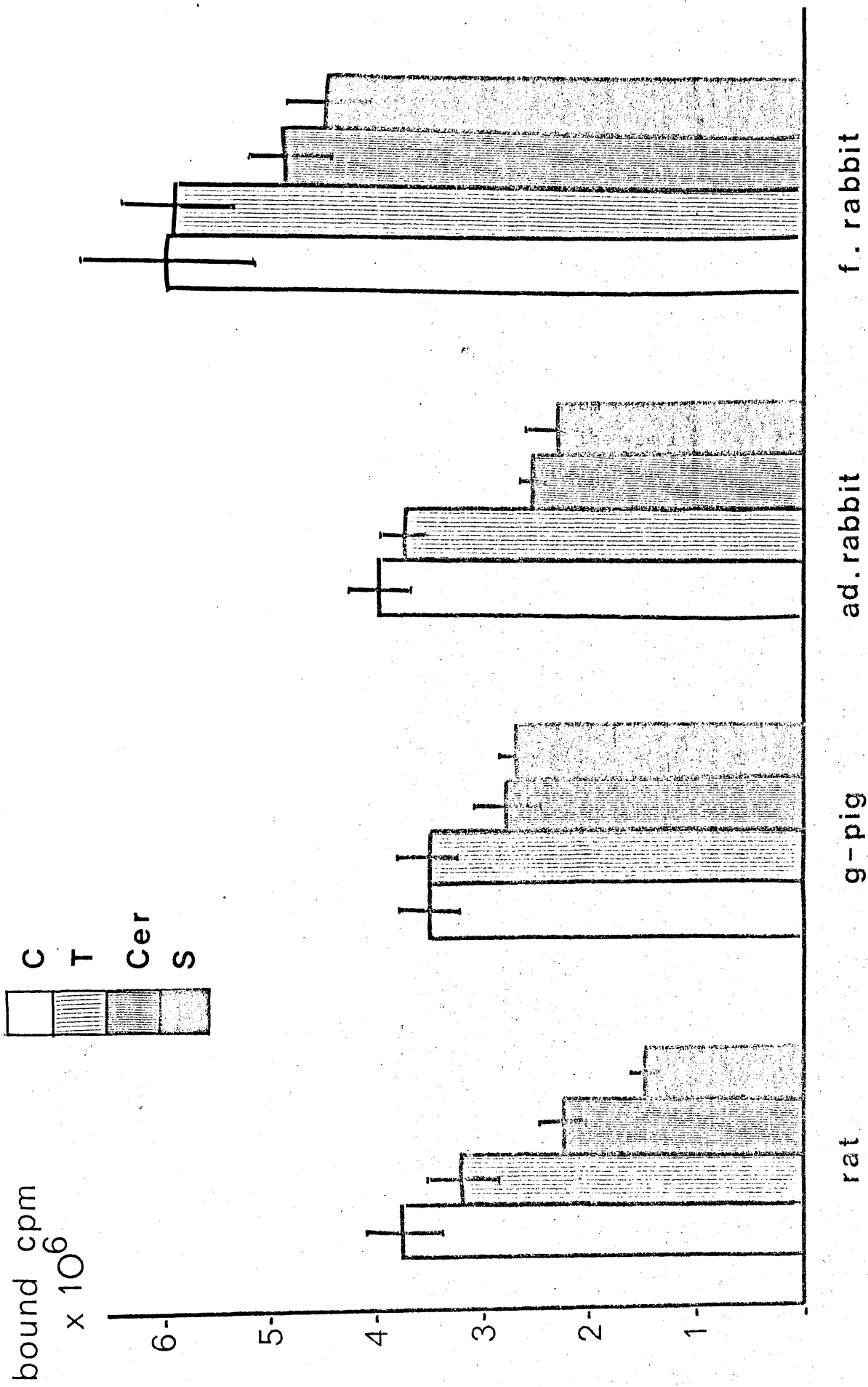


Table 3.4

Measurement of CB-activity and its distribution
in various anatomical locations of brain

Soluble and particulate extracts of brain (as indicated) were prepared as described in Chapter 2. Aliquots were removed and processed to determine CB-activity as described in Chapter 2. The data shown refer to CB-activity measured in samples obtained from 1g wet weight of each tissue, and are the means of 4 separate experiments (rat) and 2 separate experiments (guinea-pig, rabbit and foetal rabbit). Actual total wet weights of the tissues are given in the legend to Figure 3.3.

See also Table 3.3, Figure 3.4 and figure 3.5.

Table 3.4

Measurement of CB-activity and its distribution in various anatomical locations of brain

	CB-activity: bound cpm x 10 ³	% recovered CB-activity
<u>Rat</u>		
Cortex:		
soluble	2223 ± 400	60 ± 3
particulate	1467 ± 300	40 ± 3
	<u>3690</u>	
Thalamus:		
soluble	1890 ± 400	58 ± 5
particulate	1364 ± 300	42 ± 5
	<u>3254</u>	
Cerebellum:		
soluble	1400 ± 300	56 ± 6
particulate	800 ± 100	44 ± 5
	<u>2200</u>	
Brain stem:		
soluble	670 ± 150	44 ± 5
particulate	840 ± 100	56 ± 5
	<u>1510</u>	
<u>Guinea-pig</u>		
Cortex:		
soluble	1815 ± 400	51 ± 4
particulate	1705 ± 200	49 ± 4
	<u>3520</u>	
Thalamus:		
soluble	1650 ± 300	46 ± 2
particulate	1908 ± 200	54 ± 2
	<u>3558</u>	
Cerebellum:		
soluble	1410 ± 400	49 ± 6
particulate	1388 ± 150	51 ± 6
	<u>2798</u>	
Brain stem:		
soluble	750 ± 30	28 ± 3
particulate	2005 ± 400	72 ± 3
	<u>2755</u>	

Continued

Table 3.4

(Continued)

Measurement of CB-activity and its distribution in various anatomical locations of brain

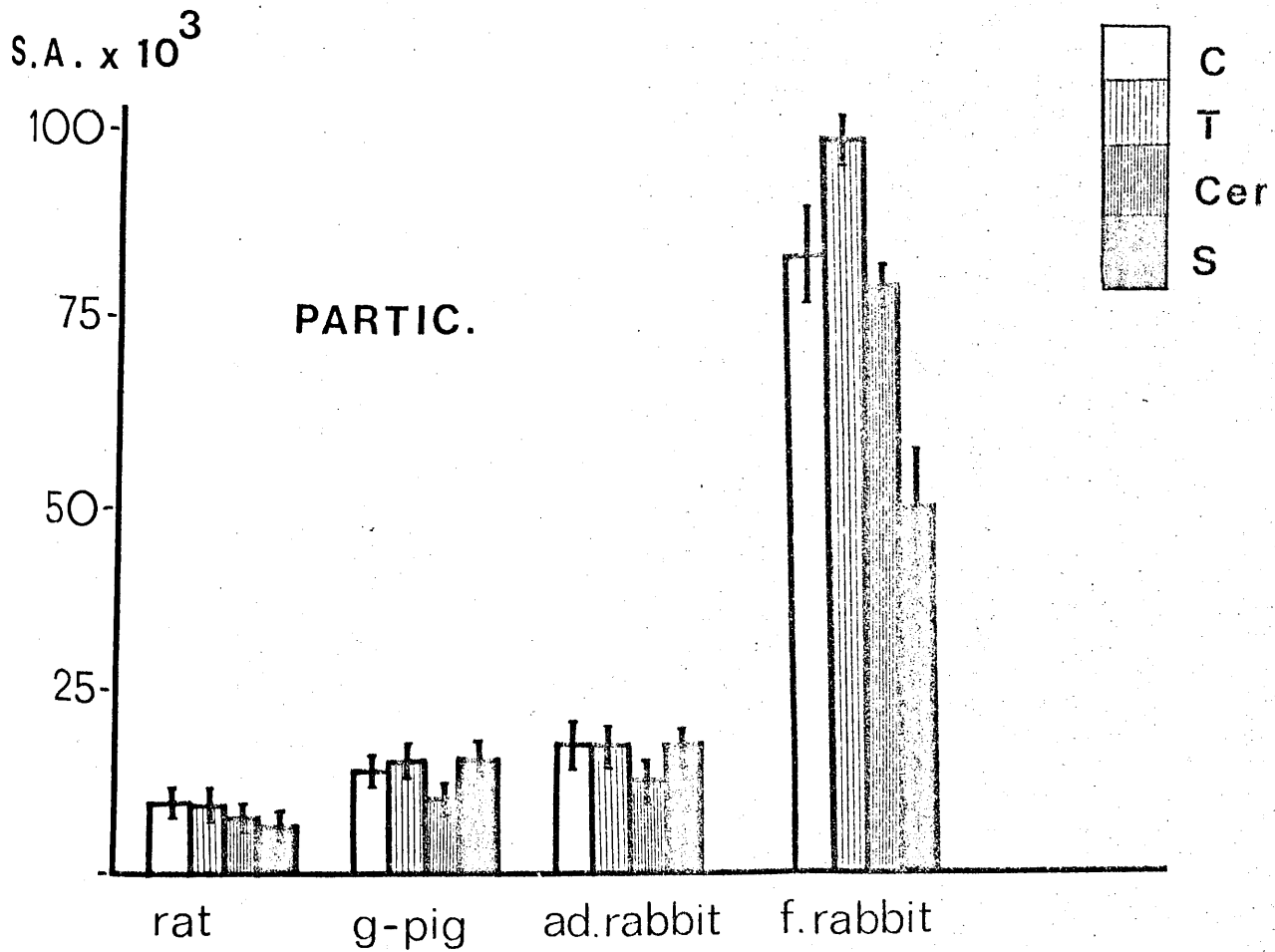
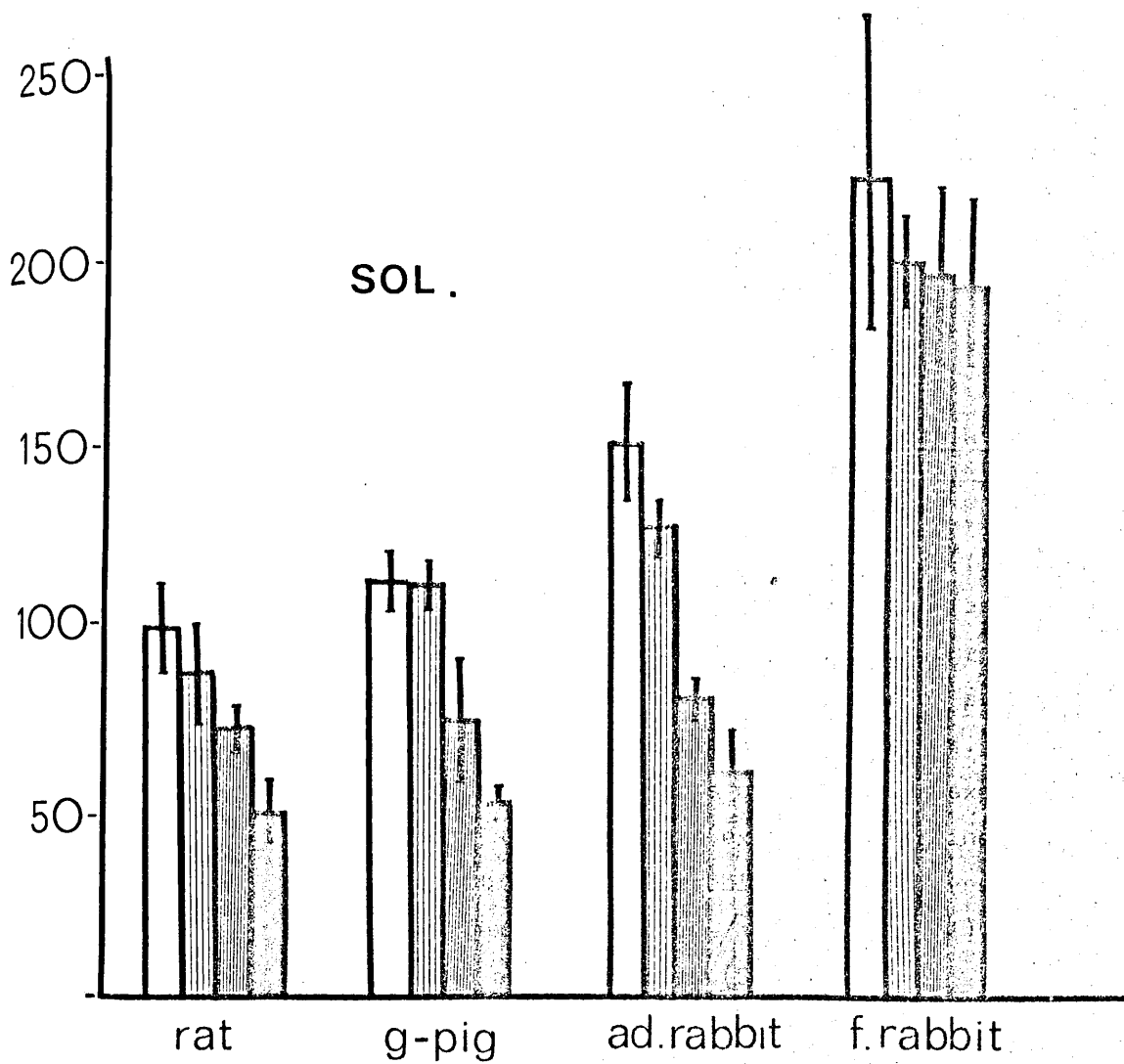
	<u>CB-activity:</u> <u>bound cpm x 10³</u>	<u>% recovered</u> <u>CB-activity</u>
<u>Rabbit</u>		
Cortex:		
soluble	2133 ± 200	54 ± 8
particulate	<u>1890 ± 400</u>	46 ± 8
	4023	
Thalamus:		
soluble	1770 ± 30	48 ± 4
particulate	<u>1950 ± 300</u>	52 ± 4
	3720	
Cerebellum:		
soluble	1300 ± 10	49 ± 2
particulate	<u>1335 ± 100</u>	51 ± 2
	2635	
Brain stem:		
soluble	688 ± 200	29 ± 2
particulate	<u>1668 ± 400</u>	71 ± 2
	2356	
<u>Foetal rabbit</u>		
Cortex:		
soluble	3250 ± 800	53 ± 14
particulate	<u>2860 ± 800</u>	47 ± 14
	6110	
Thalamus:		
soluble	2670 ± 300	46 ± 2
particulate	<u>3185 ± 600</u>	54 ± 2
	5855	
Cerebellum:		
soluble	2500 ± 300	50 ± 9
particulate	<u>2525 ± 500</u>	50 ± 9
	5025	
Brain stem:		
soluble	2333 ± 200	53 ± 9
particulate	<u>2125 ± 500</u>	47 ± 9
	4458	

Figure 3.4

Specific activities of soluble and particulate
extracts from various anatomical locations of brain

Soluble and particulate extracts of brain (as indicated) were prepared as described in Chapter 2. Aliquots were removed for determination of protein and CB-activity (as described in Chapter 2). The results given are the means of 4 separate experiments (rat) and 2 separate experiments (guinea-pig, adult rabbit and foetal rabbit). Areas investigated were: cortex (C), thalamus/hypothalamus (T), cerebellum (Ce) and brain stem (S).

See also Table 3.3, Table 3.4, Figure 3.3 and Figure 3.5.



It was a possibility that the variations of CB-activity obtained were due to differences in the inactivation rates of CB-protein during the incubation at 37° in the CB-assay, as between the various preparations. Experiments were therefore carried out on soluble preparations from different areas of adult rat brain to measure the loss in CB-activity at 37° (see Chapter 5 (f) and Fig. 5.9). It was found that CB-activity in the brain stem extract showed a faster inactivation rate than was measured in the other samples. From the rates of inactivation measured, an estimate could be made of initial CB-activity present in the samples at the beginning of the incubation procedure. The results for rat brain are given in Fig. 3.5, showing that there is ~~no~~ ^{a small but} significant difference ^{*} in the initial CB-activity present in the extracts. The basis for the faster decay rate of CB-activity in this region is unknown, but could be due to the presence of a less stable form of tubulin in the particular cell types that make up this gross anatomical region.

However, no experiments were performed to compare the rates of thermal inactivation of CB-activity between immature and adult brain, and it remains a possibility that the relatively high CB-activity in all the extracts of immature brain could partly be due to the presence of a more stable form of tubulin. This has been indicated recently in experiments in which soluble extracts of immature and adult chick brain were used (Bamburg et al, 1973).

* When the data obtained were statistically analysed, using Student's t-test, to compare CB-activity in the cortex to that present in each of the remaining areas, it was found that only the brain stem region was significantly different at $P > 0.05$ (i.e. there is a greater than 95% chance that these two areas have significantly different CB-activities). The statistical results also indicated that more experiments are required to ascertain whether significant differences exist, at this probability level, between the CB-activities measured in the remaining areas.

Figure 3.5

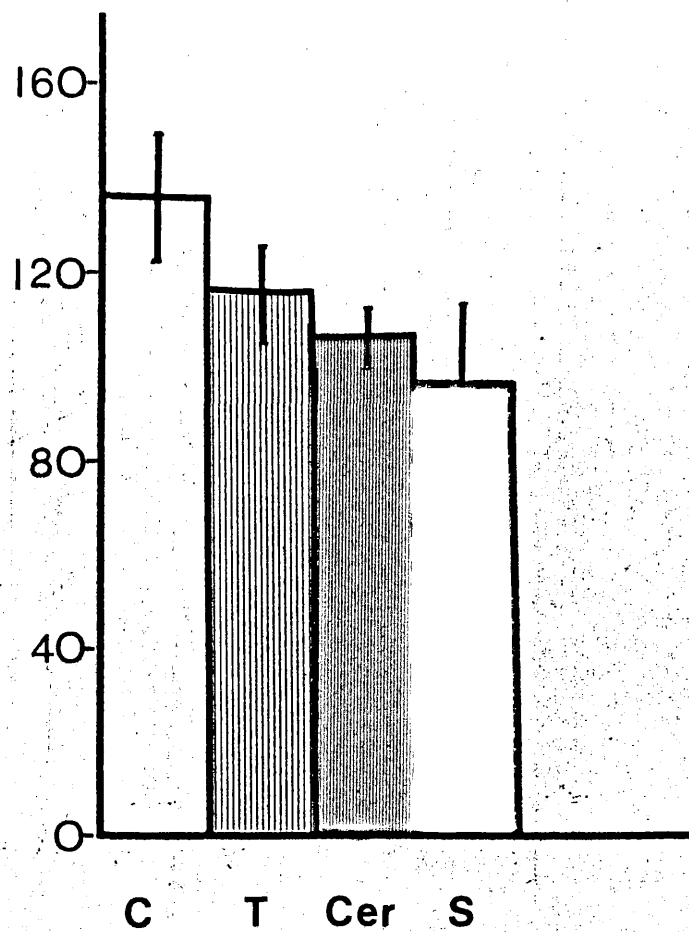
Initial specific activities of soluble fractions
obtained from various anatomical locations of
adult rat brain

Soluble extracts of adult rat brain were prepared as described in Chapter 2. They were preincubated at 37° prior to the CB-assay to determine loss in activity during the incubation with colchicine (see Chapter 5 and Figure 5.9). The initial CB-activity present in each sample was obtained by extrapolating the results to zero incubation time. Areas investigated were: cortex (C), thalamus/hypothalamus (T), cerebellum (Ce) and brain stem (S). The results are the means of two separate experiments.

Compare with Figure 3.4 which shows the specific activities of these samples with no correction for thermal inactivation.

Applying Student's t-test to these data, it was found that the measured value of CB-activity in the brain stem region was significantly different to that in the cortex at $P > 0.05$, but other significant differences did not exist between the samples at this probability level (see text).

S.A. x 10³



CHAPTER FOUR

The subcellular distribution
of colchicine-binding protein
in rat brain

Introduction

It was shown in Chapter 3 that about half the total CB-activity in buffered sucrose homogenates of brain tissue is associated with the crude particulate fraction obtained after high-speed centrifugation (10^5 g-hr). No intact microtubules are present under these conditions. This chapter deals with work on the distribution of CB-activity in primary and secondary subfractions of rat brain homogenates by two different procedures of differential and gradient centrifugation. The nature of the particulate components responsible for colchicine-binding was therefore further elucidated.

Materials and Methods

1 day old and adult rat brain was obtained as described in Chapter 2 and was used fresh to prepare a 10% homogenate in 0.32M sucrose containing 1mM sodium phosphate buffer, pH 6.8, and 1mM $MgCl_2$. Primary fractions (crude nuclear, mitochondrial, microsomal, ribosomal and soluble) and purified nuclei were prepared according to the method of Balazs and Cocks (1967). The purification of nerve-ending membranes (i.e. synaptic membranes) and vesicles from osmotically disrupted mitochondrial suspensions was carried out following the procedure of Whittaker and Sheridan (1965), and of Lapetina *et al.* (1967). The homogeneity of the subfractions was checked by electron microscopy; the purity of the nuclear fractions was checked by light microscopy and DNA determinations (as described by Burton (1956)).

All samples to be assayed for CB-activity were resuspended in, or adjusted to, 10mM sodium phosphate buffer pH 6.8, containing 10mM $MgCl_2$, and were kept on ice (maximum 3h) prior to incubation. CB-assays were performed as described in Chapter 2, incubating the protein (concentration approximately 1 mg/ml) with $2.5 \mu M$ [3H]-colchicine for 1.5h at 37° . Samples were then processed by the filter disc method to determine the amount of protein-bound radioactivity. Protein concentration was measured by the method of Lowry *et al.* (see Chapter 2 (h)). The recoveries for proteins and CB-activities were not less than 80% throughout the subcellular fractionation procedures, in fact being nearer 90% in most experiments. Electrophoresis of the subcellular fractions was performed as described in Chapter 2 using phosphate-SDS buffer and 10% acrylamide gels.

Results are expressed as specific activities (S.A.'s) which are cpm of colchicine bound/mg protein; and as relative specific activities (R.S.A.'s)

Figure 4.1

Scheme for differential centrifugation of homogenates to obtain the primary fractions

Homogenates were prepared in 9 parts of 0.32M sucrose containing 1mM sodium phosphate buffer, pH 6.8, and 1mM MgCl₂. Centrifugation of the homogenate yielded a pellet and a supernatant, which was further centrifuged as shown. The CB-activities of the fractions are given in Table 4.1 and in Figure 4.2. Results of the electrophoresis of the fractions are shown in Figure 4.3.

Figure 4.1

Scheme for differential centrifugation of homogenates to obtain the primary fractions

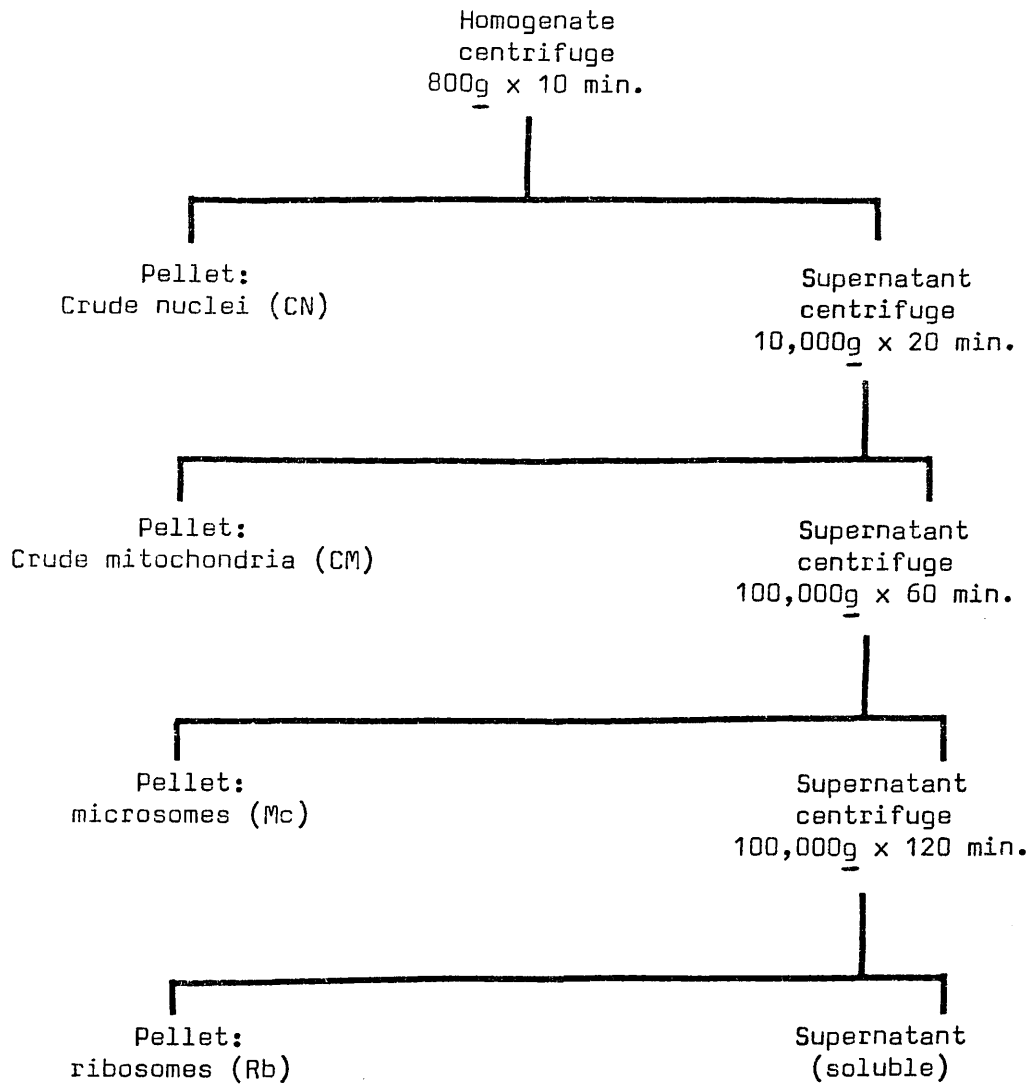


Figure 4.2

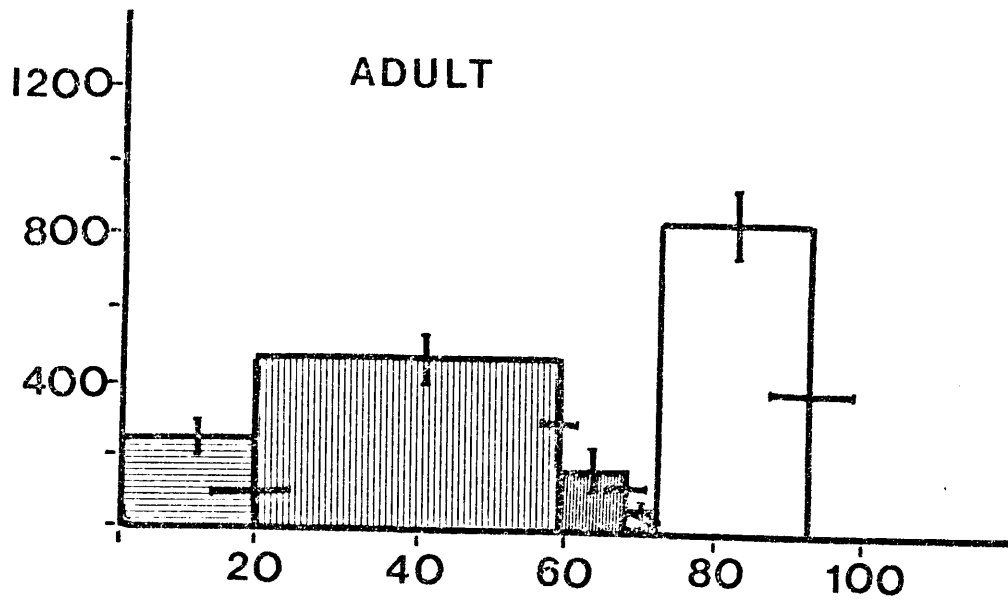
CB-activity and protein content of the primary
subcellular fractions of rat brain

Rat brain homogenates were prepared in 0.32M sucrose containing 1mM phosphate buffer, pH 6.8, and 1mM $MgCl_2$, and were centrifuged according to the scheme shown in Figure 4.1 to obtain the primary fractions. These were then assayed for CB-activity (see Chapter 2). The results refer to CB-activity measured in 1g of brain from adult or 1-day old rat and are the means of 4 separate experiments (adult) and two separate experiments (1-day).

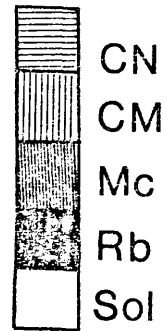
See also Table 4.1.

Results of the electrophoresis of the adult fractions are shown in Figure 4.3.

bound cpm x 10³



mg. protein



I - DAY

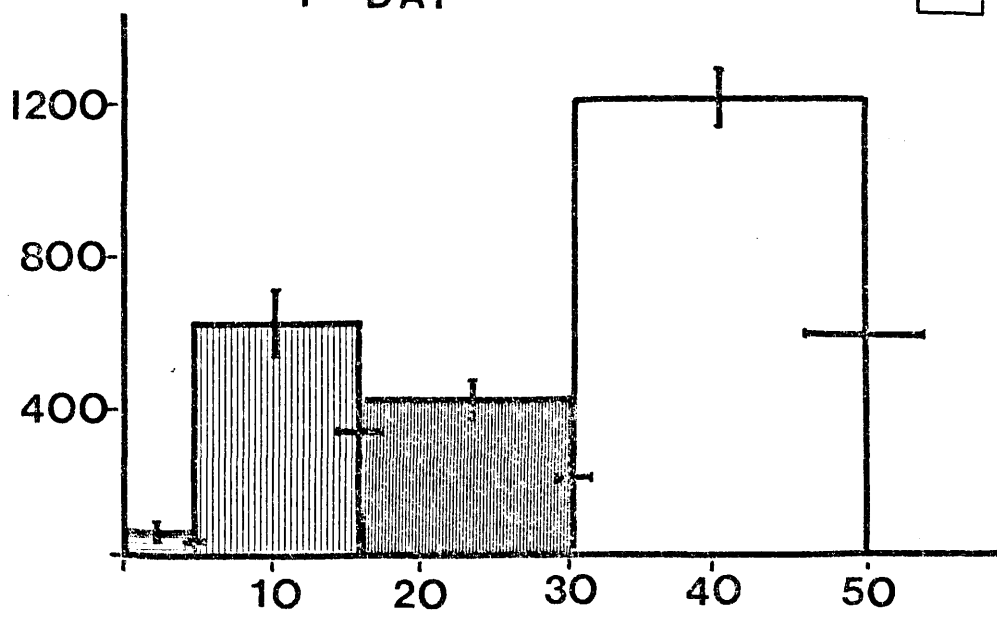


Table 4.1

Distribution of protein and CB-activity in the
primary subcellular fractions of rat brain

Homogenates were prepared in 0.32M sucrose containing 1mM phosphate buffer, pH 6.8, and 1mM $MgCl_2$, and were centrifuged according to the scheme shown in Figure 4.1 to obtain the primary fractions. These were then assayed for CB-activity (see Chapter 2). The results given are the means of 4 separate experiments (adult) and 2 separate experiments (1-day).

See also Figure 4.2.

Table 4.1

Distribution of protein and CB-activity in the primary subcellular fractions of rat brain

<u>Fraction</u>	<u>% recovered protein</u>	<u>% recovered CB-activity</u>	<u>R.S.A.</u>	<u>S.A. (x 10³)</u>
Adult:				
CN	19.2 ± 6	14.2 ± 3	0.74 ± 0.2	15 ± 5
CM	44.4 ± 2	26.2 ± 3	0.59 ± 0.1	12 ± 2
Mc	10.0 ± 3	10.0 ± 3	1.00 ± 0.2	20 ± 6
Rb	4.3 ± 1	4.0 ± 1	0.93 ± 0.2	18 ± 4
Sol	22.2 ± 6	45.6 ± 5	2.05 ± 0.3	40 ± 8
1-day:				
CN	9.1 ± 1	2.1 ± 1	0.23 ± 0.1	11 ± 2
CM	22.5 ± 4	27.5 ± 2	1.22 ± 0.2	57 ± 10
Mc	29.0 ± 3	18.4 ± 2	0.63 ± 0.1	30 ± 5
Sol	39.4 ± 8	52.0 ± 3	1.32 ± 0.2	64 ± 10

Figure 4.3

5

Electrophoresis of primary subcellular fractions
obtained from adult rat brain

The fractions were prepared as shown in the scheme in Figure 4.1. Approximately 100 μ g of protein was applied to each gel, and electrophoresis was performed in phosphate-SDS buffer as described in Chapter 2. Gels were fixed and stained with Coomassie brilliant blue. CB-activities of the fractions are given in Table 4.1.

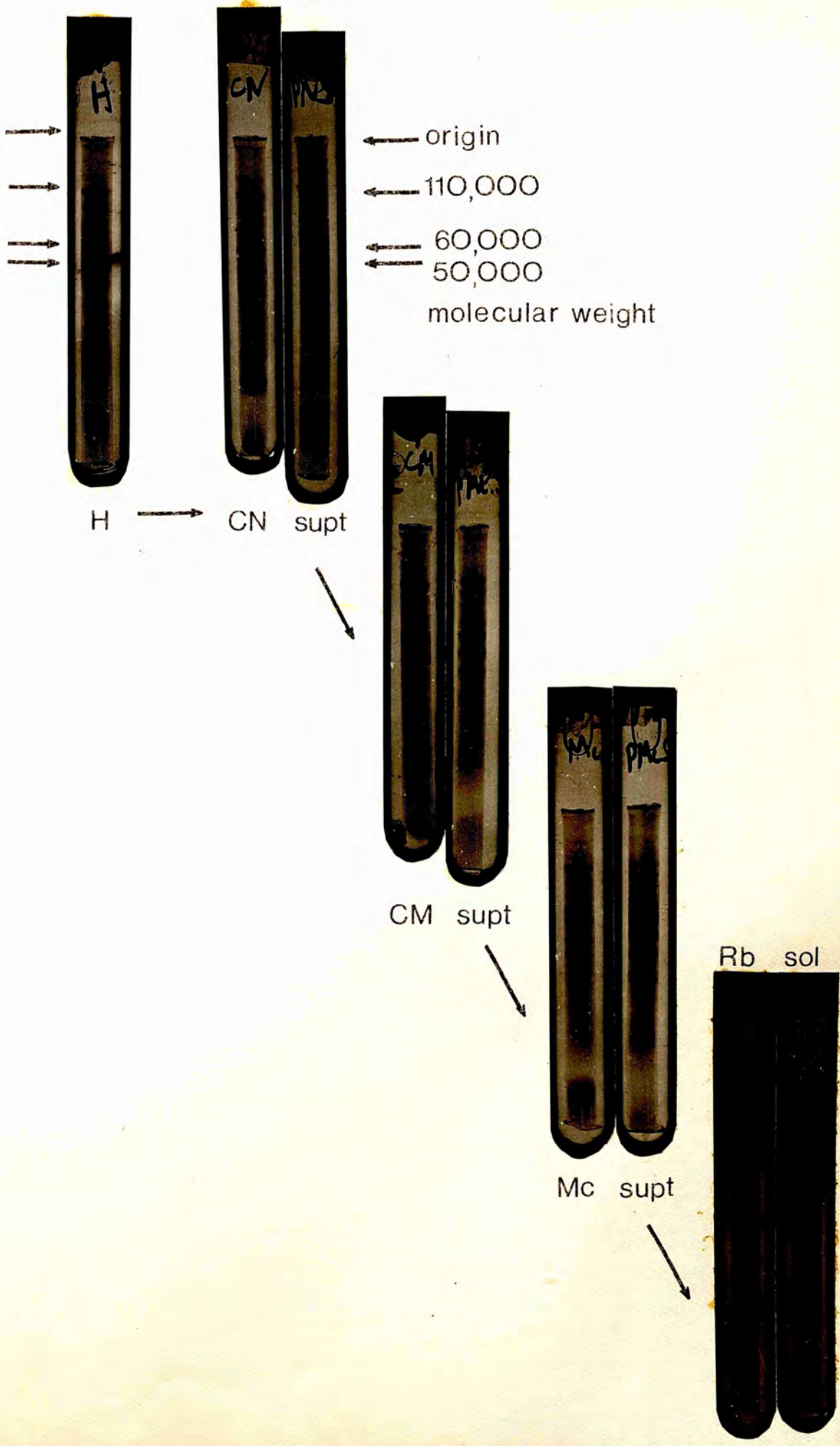
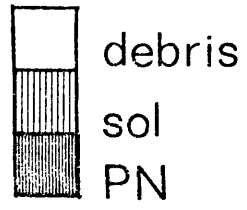


Figure 4.4

CB-activity and protein content of the nuclear subfractions obtained from adult rat brain

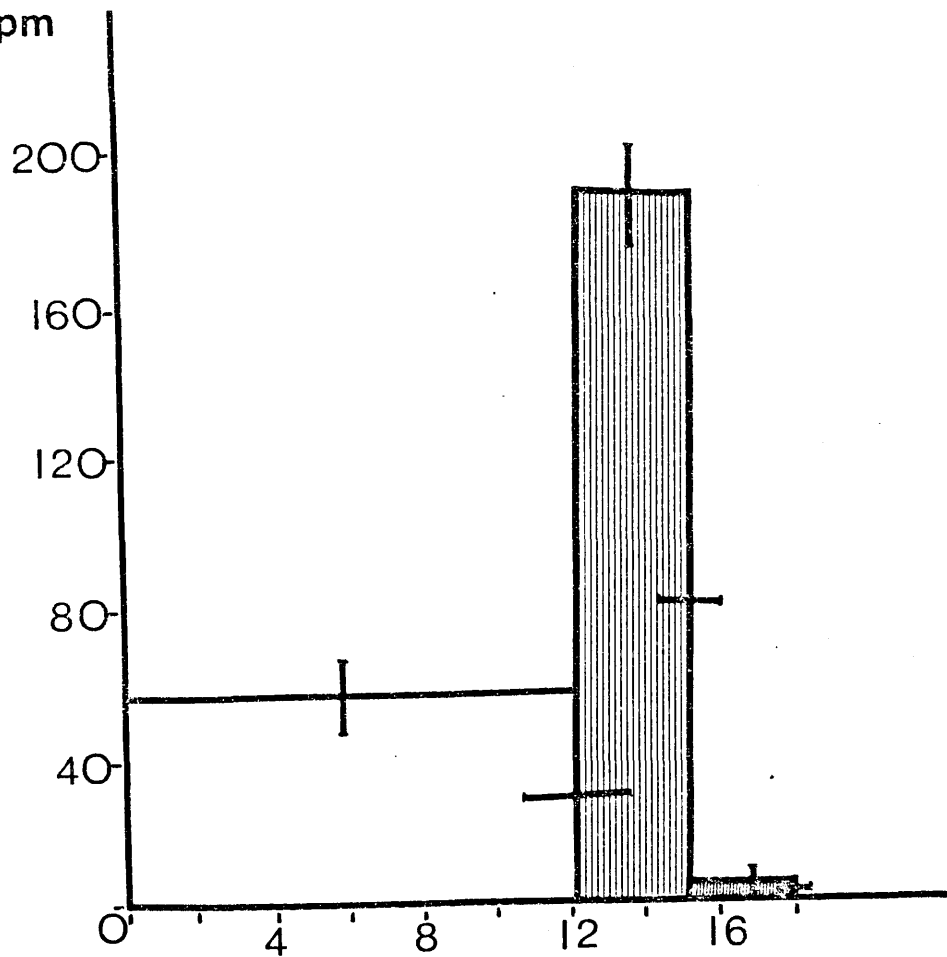
The crude nuclear fraction was prepared from adult rat brain as shown in Figure 4.1. This was re-suspended in 1.67M sucrose containing 1mM phosphate buffer and 1mM MgCl₂, and was recentrifuged for 10⁵g-h., yielding a purified nuclear pellet (PN), an intermediate soluble phase (Sol) and a floating debris layer. These subfractions were then assayed for CB-activity (see Chapter 2). The results given were obtained using 1g of brain, and are the means of two separate experiments.

See also Table 4.2.



CB-activity

(bound cpm
 $\times 10^3$)



mg. protein

Table 4.2

Distribution of protein and CB-activity in the
nuclear subfractions of adult rat brain

The fractions used were prepared and assayed for
CB-activity as described in the legend to Figure
4.4.

Table 4.2

Distribution of protein and CB-activity in the nuclear subfractions of adult rat brain

<u>Fraction</u>	<u>% recovered protein</u>	<u>% recovered CB-activity</u>	<u>R.S.A.</u>	<u>S.A. (x 10³)</u>
Debris (floating)	72.6 ± 10	22.0 ± 5	0.30 ± 0.1	5 ± 1
Soluble	17.3 ± 5	75.7 ± 10	4.38 ± 1.0	67 ± 20
PN pellet	10.1 ± 4	2.3 ± 0.5	0.23 ± 0.1	4 ± 1

which are obtained from the relation $\frac{\% \text{ recovered bound cpm}}{\% \text{ recovered protein}}$. The latter facilitates comparison of CB-activities in the various subfractions with that of the parent fraction, whose R.S.A. is taken to be 1.

Results

(a) Distribution of CB-activity in the primary fractions

Homogenates of adult and young rat brain prepared in buffered sucrose were differentially centrifuged according to the scheme shown in Fig. 4.1 to obtain the primary fractions (crude nuclear, mitochondrial, soluble and microsomal). Samples of each fraction were assayed for CB-activity and the results obtained are given in Fig. 4.2 and in Table 4.1. These show that in both the young and adult brains the fraction with the highest CB-activity was the soluble fraction. However, a considerable amount of the recovered CB-activity (about half of the total) was found associated with the particulate fractions, together with about two-thirds of the recovered protein (see also Chapter 3).

The results obtained from the electrophoresis of the primary fractions are given in Fig. 4.3, showing that each of the fractions contained proteins which migrated with a molecular weight of 60,000. It is in this region that purified tubulin also migrates.

The major part of the total particulate CB-activity recovered was located in the crude mitochondrial fractions in both adult and immature brain. Furthermore, about one-third of the particulate CB-activity in adult brain was found associated with the crude nuclear fraction. However, CB-activity in the crude nuclear fraction obtained from young brain was very low; this difference could be due to the fact that myelin, which represents only a minor constituent in immature brain, is largely responsible for the trapping of both soluble and particulate contaminants found in this fraction (Balazs and Cocks, 1967). To test this possibility the crude nuclear fraction from adult brain was further purified by re-suspending in 1.67M sucrose containing 1mM phosphate buffer pH 6.8 and 1mM MgCl_2 , and recentrifuging for 10⁵ g-h (method of Balazs and Cocks), to yield three subfractions: a purified nuclear pellet (PN), an intermediate soluble phase (Sol) and a floating 'debris' layer, each of which was assayed for CB-activity. The results shown in Fig. 4.4 and in Table 4.2 clearly indicate that trapped soluble material was responsible for the majority of the CB-activity measured in the crude nuclear fraction from adult brain. The purified nuclear fraction from adult brain was just as

low in CB-activity as was the crude nuclear fraction from young brain. DNA determinations performed showed an almost quantitative recovery of material in the purified nuclear fraction from the crude nuclear fraction, showing that contaminating material had been removed.

It was also possible that the relatively high concentration of CB-activity found in the crude microsomal and ribosomal fractions was due, in part at least, to the presence of small nerve-endings known to contaminate this fraction (Kataoka and de Robertis, 1967).

The possibility that CB-activity in the crude mitochondrial fraction was due to non-mitochondrial constituents (e.g. nerve-ending particles) was therefore next investigated.

(b) Distribution of CB-activity in mitochondrial subfractions

It can be assumed that the majority of the CB-activity found in the crude mitochondrial fraction was not due to trapped soluble material, since it has already been established that only about 10% of the total particulate CB-activity is removed when the resuspended fraction is recentrifuged (see Chapter 5). This would be largely accounted for considering the trapped soluble material present in the crude nuclear fraction (see above). The components present in the crude mitochondrial fraction include myelin, membranes, nerve-ending particles ('synaptosomes') and intact and fragmented mitochondria; these can be separated by sucrose gradient centrifugation (Whittaker, 1964). The crude mitochondrial fraction was suspended in 0.32M sucrose, layered over equal volumes of 0.8M sucrose and 1.2M sucrose, and then centrifuged for 10^5 g-h. This resulted in the separation of a top, soluble layer (in the 0.32M sucrose), a myelin and small membrane fraction (at the interface between the 0.32M sucrose and the 0.8M sucrose layers), a synaptosome (NEP) fraction (at the interface between the 0.8M sucrose and the 1.2M sucrose layers), and a purified mitochondrial pellet. The results of CB-assays on these fractions are given in Fig. 4.5 and in Table 4.3, showing that the synaptosome fraction accounted for almost half the CB-activity measured in the crude parent fraction, from both immature and adult brain. The myelin fraction obtained from immature brain contained visibly much less material than the corresponding fraction from adult brain, and was found to have a lower proportion of CB-activity. The purified mitochondrial fraction also accounted for a large proportion of the total CB-activity, but it contained an even higher proportion of the recovered protein, and on the basis of its R.S.A. value, this fraction was least enriched in CB-activity and had the lowest specific activity of all the fractions.

Figure 4.5

CB-activity and protein content of the crude
mitochondrial subfractions obtained from rat
brain

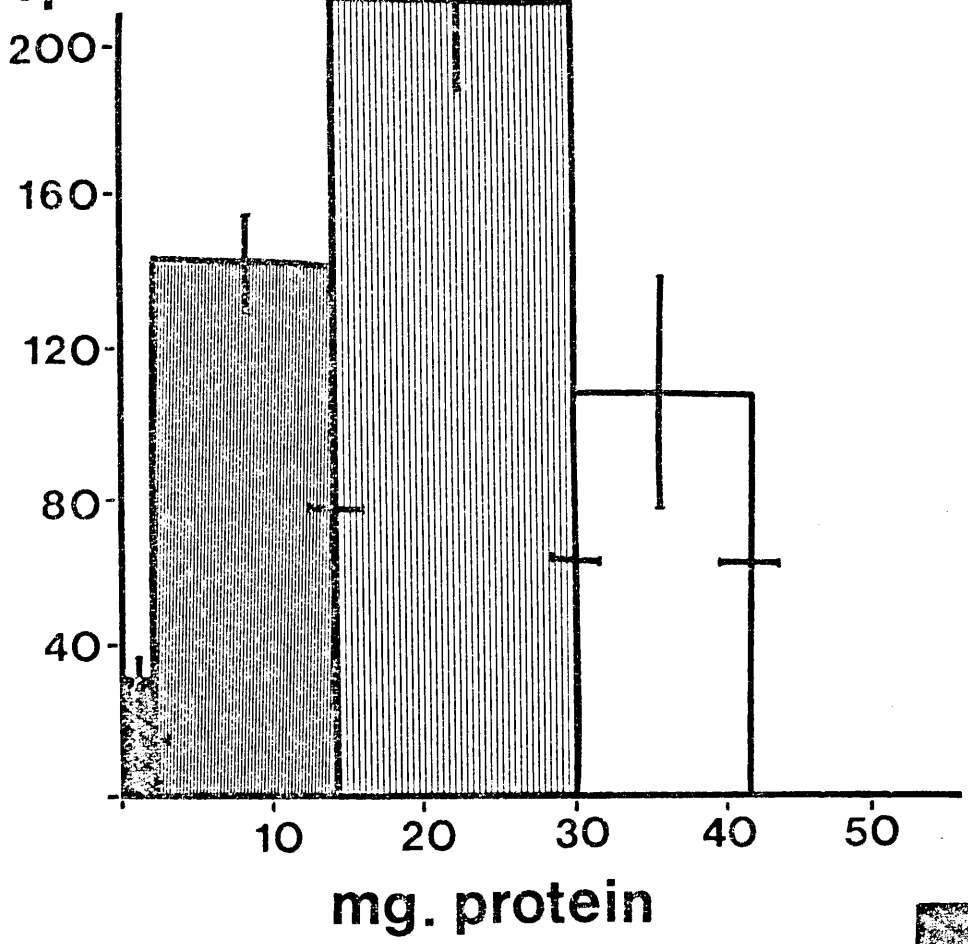
The crude mitochondrial fraction from rat brain was prepared as shown in Figure 4.1. This was resuspended in 0.32M sucrose and was layered over equal volumes of 0.8M and 1.2M sucrose, and was centrifuged for 10^5 g-h. This yielded a top (soluble) layer, a myelin + small-membrane fraction, a synaptosome (NEP) fraction and a purified mitochondrial pellet (PMT). These fractions were assayed for CB-activity (see Chapter 2). The results given were obtained using 1g of brain, and are the means of 4 separate experiments (adult) and 2 separate experiments (1-day old).

See also Table 4.3.

Results of the electrophoresis of the adult fractions are shown in Figure 4.6.

CB-activity
(bound cpm
 $\times 10^3$)

ADULT



sol
my + mer
NEP
P.Mt

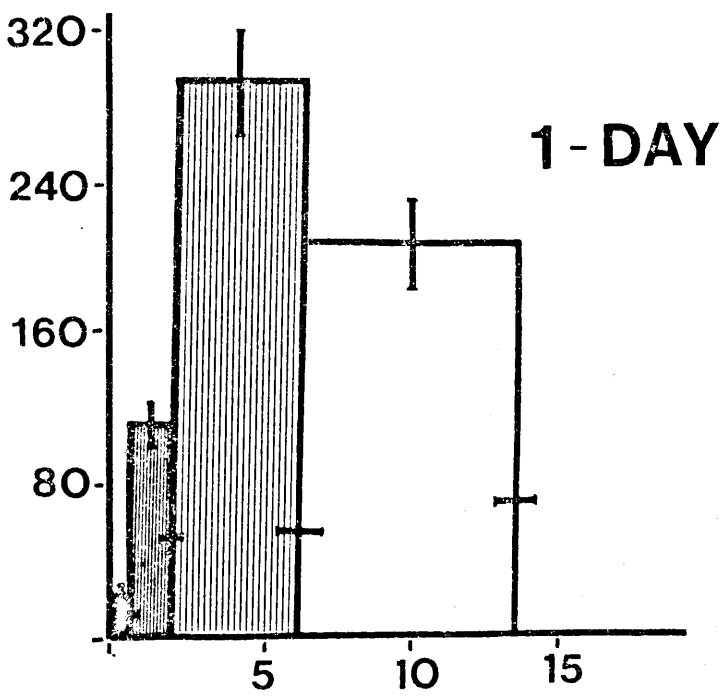


Table 4.3

Distribution of protein and CB-activity in the crude
mitochondrial subfractions from rat brain

The fractions used were prepared and assayed for
CB-activity as described in the legend to Figure 4.5.

Table 4.3

Distribution of protein and CB-activity in the crude mitochondrial subfractions from rat brain

<u>Fraction</u>	<u>% recovered protein</u>	<u>% recovered CB-activity</u>	<u>R.S.A.</u>	<u>S.A. (x 10³)</u>
Adult:				
Sol	3.6 ± 0.6	6.5 ± 1.0	1.84 ± 0.3	22 ± 4
My + Mem	27.8 ± 4.8	29.2 ± 2.7	1.07 ± 0.1	12 ± 2
NEP	38.0 ± 1.5	42.6 ± 2.6	1.12 ± 0.1	13 ± 2
P.Mt	30.6 ± 4.0	21.7 ± 5.0	0.66 ± 0.1	8 ± 1
1-day:				
Sol	3.4 ± 0.7	3.7 ± 0.6	0.98 ± 0.2	45 ± 7
My + Mem	9.9 ± 1.5	17.3 ± 1.7	1.75 ± 0.1	87 ± 10
NEP	36.0 ± 2.9	46.6 ± 4.6	1.30 ± 0.1	60 ± 6
P.Mt	50.7 ± 7.3	32.8 ± 3.9	0.65 ± 0.1	30 ± 5

The results obtained from the electrophoresis of the crude mitochondrial subfractions are given in Fig. 4.6, showing the presence of tubulin-comigrating protein in all the subfractions analysed. This would be consistent with the distribution of CB-activity observed, although no quantitative conclusions can be made relating tubulin-comigrating protein on gels and CB-protein (e.g. the purified mitochondrial fraction shows the presence of a fairly large band in the 60,000 M.Wt. region, but this fraction was shown to have a low CB-activity).

It was now evident that the synaptosome fraction made an important contribution to particulate CB-activity. Synaptosomes are pinched-off nerve endings to which post-synaptic membranes and their associated specialisations often remain attached, and they contain mitochondria, vesicles (400-600Å diameter), and some cytoplasm and smooth membranes. These constituents can be released from the intact structure by subjecting the washed synaptosome preparation to osmotic shock, bursting the synaptosome membrane. This was done simply by resuspending the fraction in distilled water only, at neutral pH, and leaving the sample to stand (lyse) at 4° for about an hour. The lysate was then centrifuged at 10,000g for 20 min. to sediment any remaining intact structures. The supernatant, which contained the contents of the synaptosomes, was layered (0.6 ml samples) over 0.4M sucrose (0.8 ml) and 0.6M sucrose (2.6 ml) and was centrifuged for 10⁵g-h. Four subfractions were obtained: a top aqueous layer (NEP-soluble), a vesicle fraction (in the 0.4M sucrose), a small-membrane fraction (at the interface between the 0.4M and 0.6M sucrose), and a pellet which contained the synaptosome mitochondria and large membrane fragments. The results of CB-assays on these fractions are given in Fig. 4.7 and in Table 4.4, showing that the small-membrane fraction had the highest CB-activity, also an almost two-fold enrichment of CB-activity compared to the parent synaptosome fraction.

Since the membrane fraction made an important contribution to the CB-activity measured in synaptosome preparations, a membrane fraction was next prepared from the crude mitochondrial fraction, and its CB-activity investigated. A crude mitochondrial fraction was prepared as described in Fig. 4.1 and this was disrupted by osmotic shock by resuspending in water (pH 7.0) at 4°. After standing for about an hour, the suspension was centrifuged at 20,000g for 30 min. to pellet the mitochondria and large membrane fragments. Small fragments of membranes, vesicles and soluble material remained in the supernatant. The pellet and supernatant were further fractionated as shown schematically in Fig. 4.8 to separate the

Figure 4.6

Electrophoresis of the mitochondrial subfractions
from adult rat brain

The subfractions were prepared as described in the legend to Figure 4.5. Approximately 100 μ g of protein was loaded per gel and electrophoresis was carried out in phosphate-SDS buffer (as described in Chapter 2). Gels were fixed, then stained with Coomassie brilliant blue.

The CB-activities of the subfractions are given in Figure 4.5 and in Table 4.3.

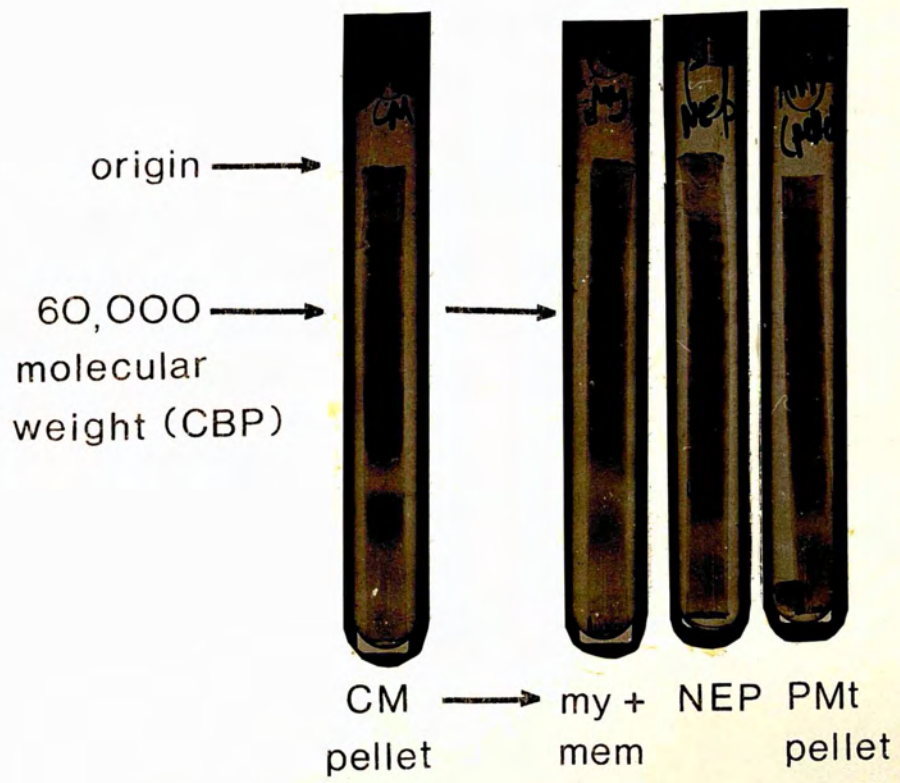


Figure 4.7

CB-activity and protein content of the lysed
synaptosome subfractions from adult rat brain

A synaptosome (NEP) preparation was obtained as described in the legend to Figure 4.5. This was lysed by suspending it in water and the sample was clarified by centrifugation. The sample was fractionated by sucrose gradient centrifugation by layering it over 0.4M and 0.6M sucrose and centrifuging for 10^5 g-h. This yielded four sub-fractions: an aqueous soluble layer, a vesicle fraction, a small-membrane fraction and a NEP-mitochondria and large-membrane pellet. These samples were assayed for CB-activity. The results given are the means from 2 separate experiments, and were obtained starting with 1g of brain in the preparation.

See also Table 4.4.

¹²⁵I-B-activity

bound cpm x 10³)

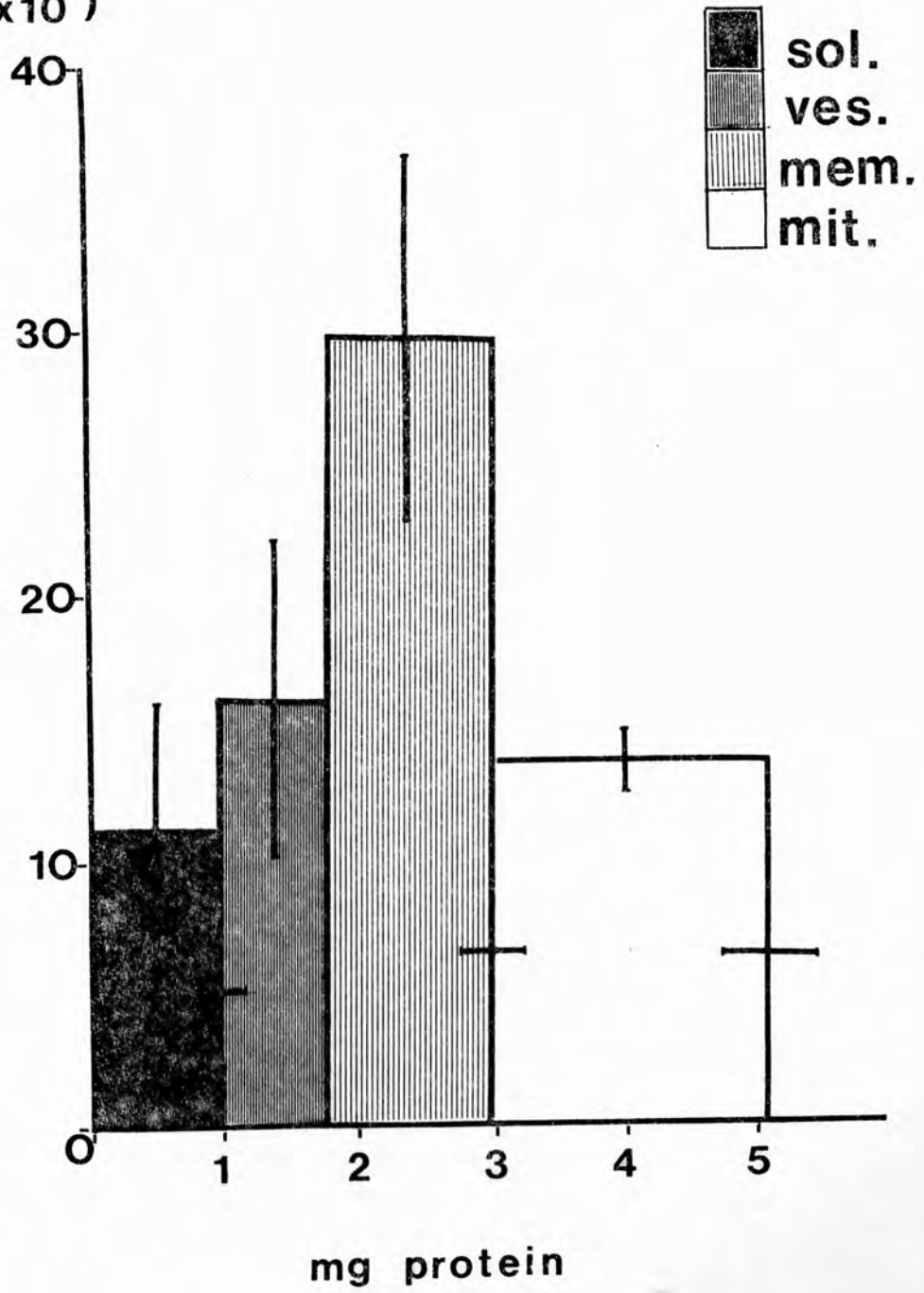


Table 4.4

Distribution of protein and CB-activity in the lysed
synaptosome subfractions from adult rat brain

The fractions used were prepared and assayed for CB-activity as described in the legend to Figure 4.7.

Table 4.4

Distribution of protein and CB-activity in the lysed synaptosome subfractions from adult rat brain

<u>Fraction</u>	<u>% recovered protein</u>	<u>% recovered CB-activity</u>	<u>R.S.A.</u>	<u>S.A. ($\times 10^3$)</u>
Soluble	17.5 \pm 2.5	14.7 \pm 3.5	0.83 \pm 0.1	12 \pm 4
Vesicles	16.2 \pm 0.1	22.0 \pm 2.9	1.36 \pm 0.2	20 \pm 8
Membranes	25.0 \pm 0.5	44.0 \pm 2.0	1.77 \pm 0.1	25 \pm 6
Mitochondria	41.3 \pm 2.5	19.3 \pm 4.5	0.46 \pm 0.1	6 \pm 1

Figure 4.8

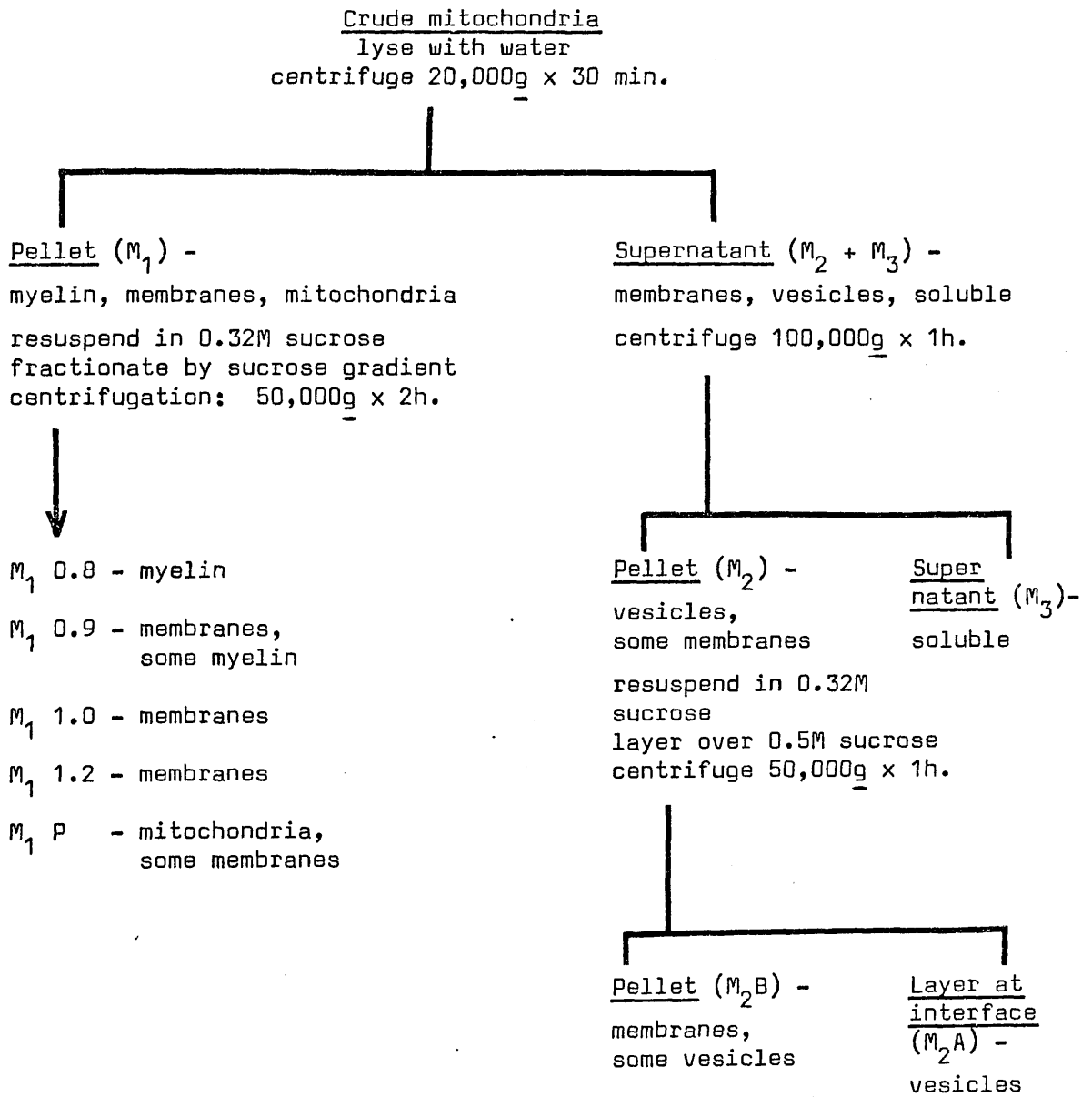
Scheme for subfractionation of water-lysed crude mitochondria from adult rat brain

The crude mitochondrial fraction was prepared as described in Figure 4.1, and was subfractionated after osmotic shock according to the method of Lapetina et al (1967) as outlined in the Figure. A photograph of the gradient of M_1 obtained is shown in Figure 4.9. The vesicles obtained in fraction M_2A are shown in the electron micrograph Figure 4.10. The CB-activities of the subfractions are given in Table 4.5 and in Figure 4.11.

Results of the electrophoresis of fractions M_1 , M_2A , M_2B and M_3 are shown in Figure 4.12.

Figure 4.8

Scheme for subfractionation of water-lysed crude mitochondria from adult rat brain



constituents they contained. A photograph of the gradient obtained in the membrane (M_1) subfractionation is shown in Fig. 4.9. The vesicles obtained (in the fraction M_2A) are shown in the electron micrograph in Fig. 4.10. The results of CB-assays of these fractions are given in Fig. 4.11 and in Table 4.5, from which it can be seen that the bulk of the CB-activity was recovered in the membrane fraction M_1 . When this was further fractionated by sucrose gradient centrifugation, the fractions obtained which contained predominantly synaptic membranes ($M_10.9$, $M_11.0$, $M_11.2$) showed a considerable enrichment of CB-activity (R.S.A. 1.30-1.65). Taken together, the fractions rich in synaptic membranes account for 65% of the CB-activity of M_1 , or about half the activity originally present in the crude mitochondrial fraction. This represents a considerable degree of localisation of CB-protein in synaptic membranes, confirming the results obtained after fractionation of synaptosome preparations (see Table 4.4).

The results obtained from the electrophoresis of these fractions are given in Fig. 4.12, which again shows the presence of a tubulin-comigrating protein band in each of the samples. It is interesting that the proportion of this protein in the fractions seems to compare reasonably with the R.S.A. values obtained, indicating a possible quantitative comparison with CB-protein in cell extracts prepared in this way.

Figure 4.9

Photograph of the sucrose gradient produced from the membrane fraction (M_1) obtained from a lysed crude mitochondrial preparation

Fraction M_1 was prepared as outlined in Figure 4.8, and was suspended in 0.32M sucrose and layered on the gradient prepared as shown, using 5ml of each sucrose concentration. The gradient was centrifuged at 50,000g for 2h. at 4°, producing several purified membrane subfractions of varying sizes. The CB-activities of the subfractions are given in Table 4.5 and in Figure 4.11.

sucrose
conc.

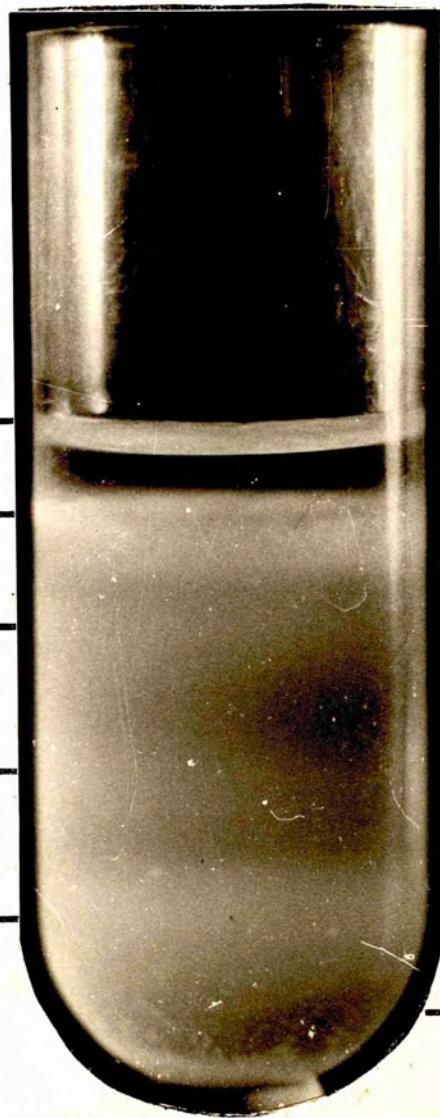
0.32 M

0.8 M

0.9 M

1.0 M

1.2 M



fraction

M₁ 0.8

M₁ 0.9

M₁ 1.0

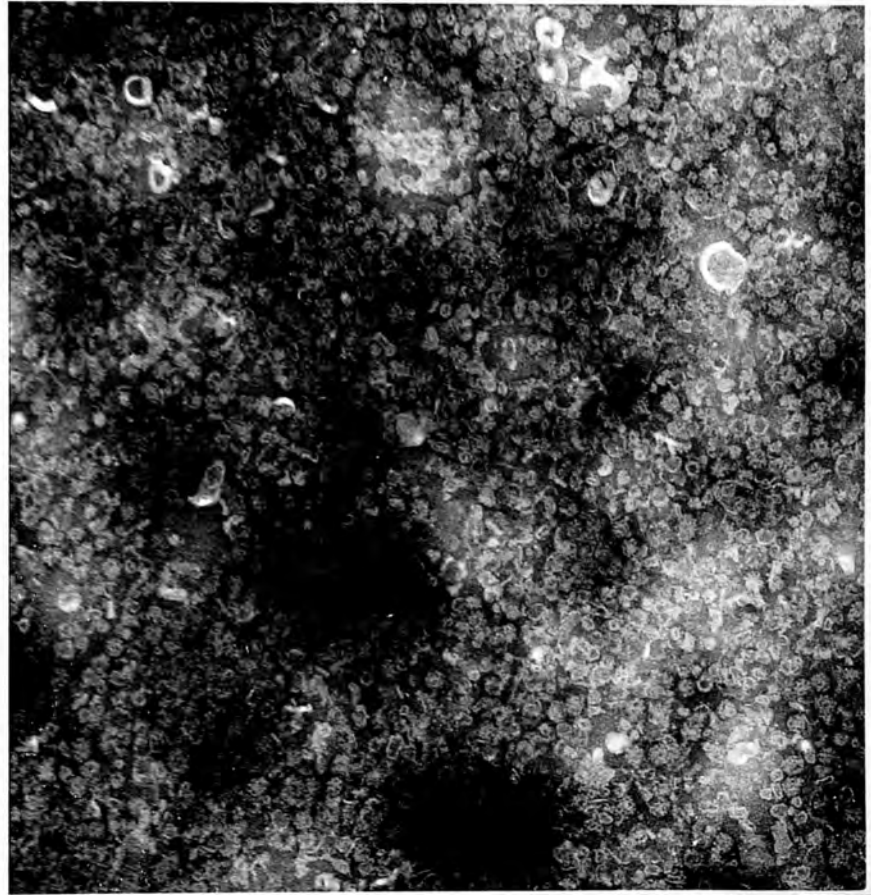
M₁ 1.2

M₁ P

Figure 4.10

Electron micrograph of the vesicle fraction (M_2A)
obtained from a lysed crude mitochondrial
preparation

Fraction M_2A was prepared as outlined in Figure 4.8 and was fixed with 3% glutaraldehyde and negative stained.



—
0.1 μ
(1000Å)

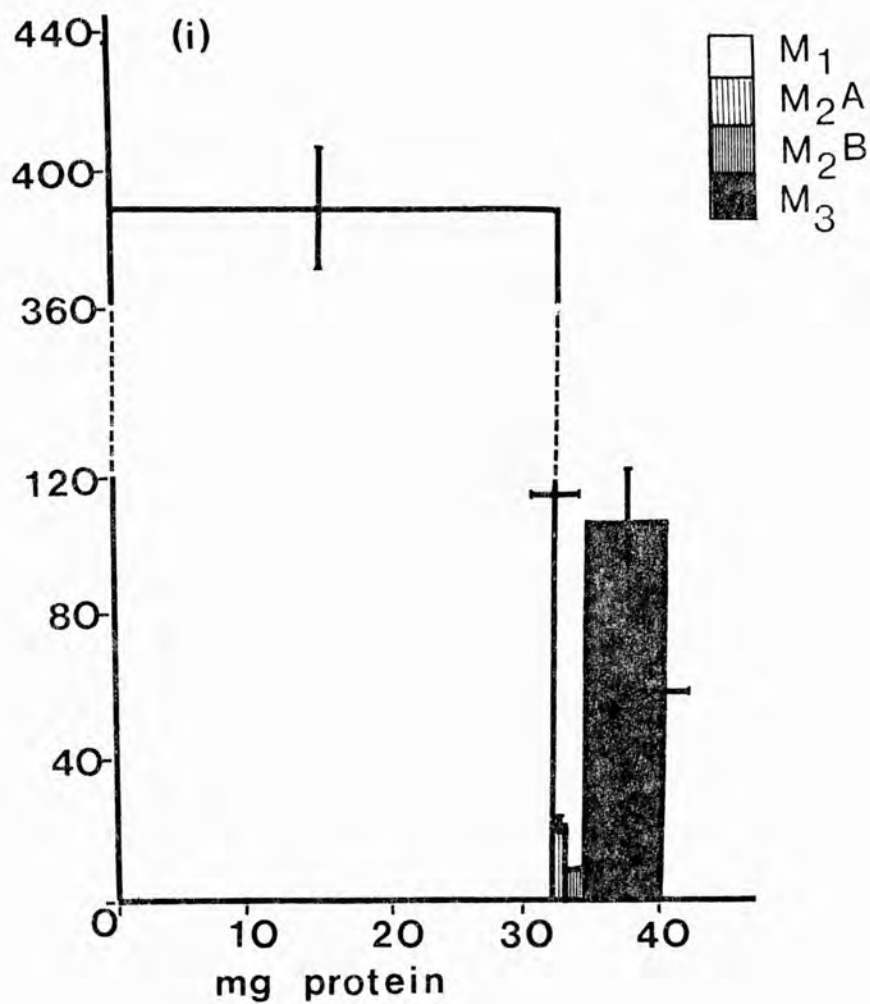
Figure 4.11

CB-activity and protein content of the subfractions
obtained from water-lysed crude mitochondria from
adult rat brain

The fractions were prepared according to the scheme shown in Figure 4.8. They were assayed for CB-activity (see Chapter 2). The results given are the means of 4 separate experiments (2 separate experiments for the M_1 subfractionation) and were obtained starting with 1g of brain in the preparation.

See also Table 4.5.

CB-activity
(bound cpm x 10³)



CB-activity
(bound cpm x 10³)

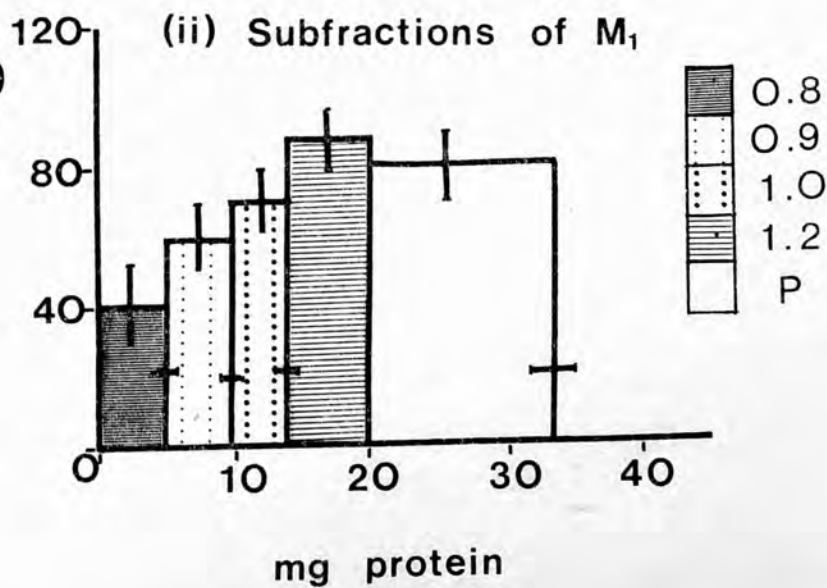


Table 4.5

Distribution of protein and CB-activity in the subfractions obtained from water-lysed crude mitochondria from adult rat brain

The fractions used were prepared as described in Figure 4.8 and were assayed for CB-activity (see Chapter 2).

See also Figure 4.11.

Results of the electrophoresis of the fractions are shown in Figure 4.12.

Table 4.5

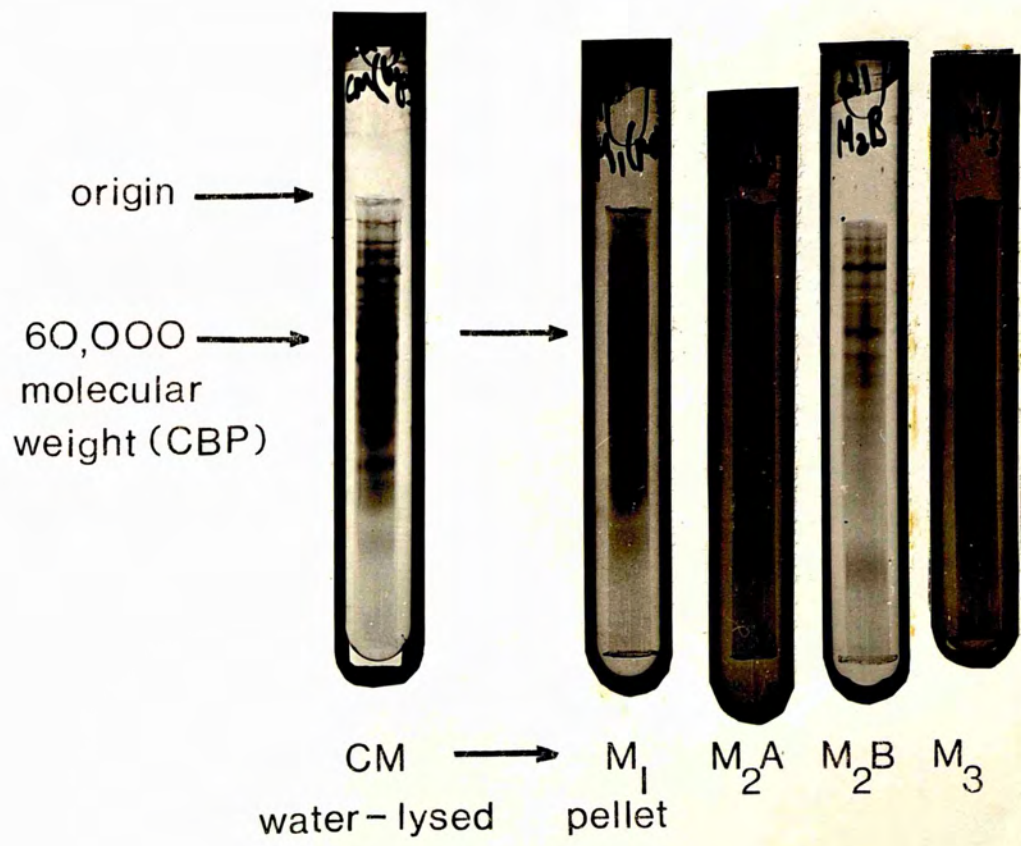
Distribution of protein and CB-activity in the subfractions obtained from water-lysed crude mitochondria from adult rat brain

<u>Subfraction</u>	<u>% recovered protein</u>	<u>% recovered CB-activity</u>	<u>R.S.A.</u>	<u>S.A. ($\times 10^3$)</u>
(i) M ₁	75.6 \pm 4.0	77.1 \pm 4.4	1.03 \pm 0.1	13 \pm 1
M ₂ A	3.8 \pm 0.2	3.4 \pm 0.2	0.89 \pm 0.1	18 \pm 5
M ₂ B	2.8 \pm 0.1	0.8 \pm 0.1	0.29 \pm 0.1	7 \pm 3
M ₃	17.8 \pm 0.2	18.7 \pm 0.1	1.05 \pm 0.1	22 \pm 8
(ii) M ₁ 0.8	15.2 \pm 1.2	12.1 \pm 3.0	0.80 \pm 0.2	8 \pm 2
M ₁ 0.9	13.6 \pm 0.9	17.7 \pm 3.0	1.30 \pm 0.2	12 \pm 2
M ₁ 1.0	12.6 \pm 0.9	20.9 \pm 3.5	1.65 \pm 0.3	17 \pm 4
M ₁ 1.2	18.2 \pm 1.2	26.0 \pm 3.5	1.43 \pm 0.3	15 \pm 3
M ₁ P	40.5 \pm 6.0	23.4 \pm 3.0	0.58 \pm 0.2	6 \pm 2

Figure 4.12

Electrophoresis of water-lysed mitochondrial subfractions from adult rat brain

The subfractions were prepared as outlined in Figure 4.8. Approximately 100 μg of protein was loaded on to each gel and electrophoresis was carried out in phosphate-SDS buffer (as described in Chapter 2). Gels were fixed, then stained with Coomassie brilliant blue. The CB-activities of these subfractions are given in Figure 4.11 and in Table 4.5.



CHAPTER FIVE

Studies on some biochemical
properties of CB-protein

Introduction

In this Chapter some of the biochemical properties and CB-characteristics of presumed microtubule subunit protein found in the soluble and particulate fractions of rat brain homogenates were investigated. The results obtained are compared in an effort to find if these two forms of CB-protein have similar properties (i.e. if they contain closely related CB-proteins) with respect to their ability to bind colchicine, rate of inactivation, and effects of stabilising agents.

The crude particulate fraction used is known to contain many different types of particles (see Chapter 4). Of these, the synaptic membrane fraction makes an important contribution to the total CB-activity. A purified synaptic membrane preparation was therefore made, and some of its properties were also investigated, and compared to those of the total soluble fraction.

Attempts were also made to solubilise the CB-protein associated with the crude particulate fraction and these are summarised at the end of the Chapter. The harshness of conditions needed to release the protein would reflect the strength with which the protein is bound to other constituents of this fraction. Once isolated, the properties of this membrane-bound CB-protein could be investigated and its characterisation begun.

Materials and Methods

Soluble and crude particulate fractions of rat brain were prepared from homogenates in 0.32M sucrose containing 10mM sodium phosphate buffer, pH 6.8, and 10mM $MgCl_2$, as described in Chapter 2 (b).

The CB-activity of the samples was determined by the filter disc method, as described in Chapter 2 (d), unless otherwise stated in the text (e.g. when the protein concentration, colchicine concentration, incubation time or temperature were varied).

A phosphate-glutamate buffer system used to assay CB-activity (Wilson, 1970) was tested in some experiments. This was prepared as follows: 20mM sodium phosphate buffer, pH 6.8, was made by diluting the stock phosphate buffer (see Chapter 2). Sodium glutamate was dissolved in this directly to give a concentration of 100mM.

Purified synaptic membrane preparations were isolated from the crude mitochondrial fraction as described by Cotman et al (1971). Rat brains were homogenised in 4 volumes of ice-cold isolation medium (0.25M sucrose, containing 0.01M tris-HCl buffer, pH 7.4, and 0.05mM dipotassium EDTA) and this was centrifuged at 2,000g for 3' to pellet intact cells.

The supernatant was centrifuged at $12,500g$ for 8' to pellet the mitochondria and synaptosomes. The pellet was resuspended, using a Teflon rod, in a 3% Ficoll medium (also containing 0.24M D-mannitol, 0.06M sucrose and 0.05mM EDTA, made to pH 7.4 with tris) using about 1 ml/g brain. Up to 10 ml of this sample was then layered over 25 ml of a 6% Ficoll medium (containing 0.24M D-mannitol, 0.06M sucrose and 0.05mM EDTA, made to pH 7.4 with tris) and this was centrifuged at $11,500g$ for 30'. A pellet (purified mitochondria) was obtained, together with a floating layer (myelin) and material at the interface of the two Ficoll layers (this contains the synaptosomes). This layer was pipetted out, diluted at least 1:1 with isolation medium, and centrifuged at $18,000g$ for 30' to pellet the synaptosomes. These were then lysed by resuspending in 6mM tris-HCl buffer, pH 8.1, (about 4 ml/g brain) and standing for about 1-1½ hours in the cold. The synaptic membranes were then pelleted by centrifuging at $18,000g$ for 15', and were resuspended in 10mM tris-HCl buffer, pH 7.4, containing 0.32M sucrose and 1mM EDTA (about 1 ml/3g brain). The membranes were then purified by gradient centrifugation, layering a 1 ml sample over 1.5 ml of a 7.5% Ficoll medium (also containing 0.32M sucrose and 1mM EDTA in 0.01M tris-HCl buffer, pH 7.4) and 1.5 ml of a 13% Ficoll medium (also containing 0.32M sucrose and 1mM EDTA in 0.01M tris-HCl buffer, pH 7.4). These samples were centrifuged at $96,000g$ for 30', when the purified membrane fraction could be collected at the top interface (the 0.32M sucrose-7.5% Ficoll, 0.32M sucrose interface). This fraction was then diluted with isolation medium and pelleted, to free it of any contaminating Ficoll medium. In this preparation, 10g of brain yielded about 10-15mg of synaptosomal membrane protein.

Rat brain particulate preparations bind considerably less colchicine/mg protein than do soluble preparations. In experiments in which the colchicine concentration was decreased well below the $2.5 \mu\text{M}$ usually used, a significantly measurable level of bound colchicine was therefore not obtained. Consequently, a colchicine solution of higher specific radioactivity was used, and this was made by omitting the unlabelled colchicine from the stock colchicine solution (see Chapter 2 (f)). The original [^3H]-colchicine solution (approximately $500 \mu\text{M}$) was diluted 10 times to make a stock $50 \mu\text{M}$ solution. The specific radioactivity of this stock solution was $100,000 \text{ cpm}/\mu\text{l}$ (at 48% efficiency this corresponds to $2,000 \text{ cpm}/\text{pmole}$). When diluted 20 times in the incubation mixtures ($25 \mu\text{l}$ in 0.5ml), the final colchicine concentration obtained was $2.5 \mu\text{M}$. In experiments in which this higher specific radioactivity colchicine was used, the results are expressed in terms of pmoles of colchicine bound to correct for this difference.

In other experiments, results are expressed as specific activities (S.A.'s) which are cpm bound colchicine/mg protein.

Results

(a) Colchicine binding as a function of colchicine concentration

Soluble and particulate preparations were incubated in 10mM sodium phosphate buffer, pH 6.8, containing 10mM MgCl₂ (protein concentration up to 1mg/ml) with various concentrations of [³H]-colchicine (final concentration 0.25 to 10 μM) for 1.5h at 37°C. Samples were then processed by the filter disc assay (see Chapter 2), to determine the amount of protein-bound radioactivity. The results, shown graphically in Fig. 5.1, indicate that in each preparation, half-maximal binding was obtained using approximately 2 μM colchicine.

If a reciprocal plot is made, an estimate of the dissociation constant for the binding reaction can be made (see Fig. 5.2). For each of the samples tested, the constant obtained was within a very narrow range (2 to 3 μM), indicating that in this property the CB-proteins from the different preparations showed very similar behaviour.

(b) Colchicine binding as a function of protein concentration

Soluble and particulate preparations (protein concentrations varying up to 1.4 mg/ml) were assayed for CB-activity and the results obtained are shown graphically in Fig. 5.3. It is apparent that the amount of colchicine bound is proportional to the protein concentration within the range used. In practice, therefore, a protein concentration of up to 1 mg/ml was routinely used.

(c) Colchicine binding as a function of incubation time and temperature

Soluble and particulate preparations were incubated with 2.5 μM [³H]-colchicine for various times at temperatures ranging from 2°C to 55°C. Samples were then processed by the filter disc assay (see Chapter 2), to determine the amount of protein-bound radioactivity. The results, shown graphically in Figs. 5.4 and 5.5, show that maximum binding occurs at 37°C after 1.5 to 3h incubation for both types of preparation. It also appears that the maximum binding occurs within a much narrower temperature range for the soluble than for the particulate preparation. It is a possibility that this is because the relative stability of the particulate CB-protein at higher temperatures may be due to increased protection of colchicine-binding sites when these are associated with membrane components. It is clear from subsequent experiments that particulate CB-sites are very firmly bound to membranes (this Chapter, section (k)).

Figure 5.1

Colchicine-binding as a function of colchicine concentration

Soluble and particulate extracts of brain were prepared as described in Chapter 2. Synaptic membranes were prepared by the method of Cotman et al (see Methods). Binding mixtures were set up containing protein (concentration up to 1mg/ml) and varied colchicine concentrations (0.25 to 10 μ M), and were incubated at 37^o for 1.5h. then were processed by the filter disc assay (see Chapter 2) to measure amount of protein-bound radioactivity. A reciprocal plot of these data is shown in Figure 5.2.

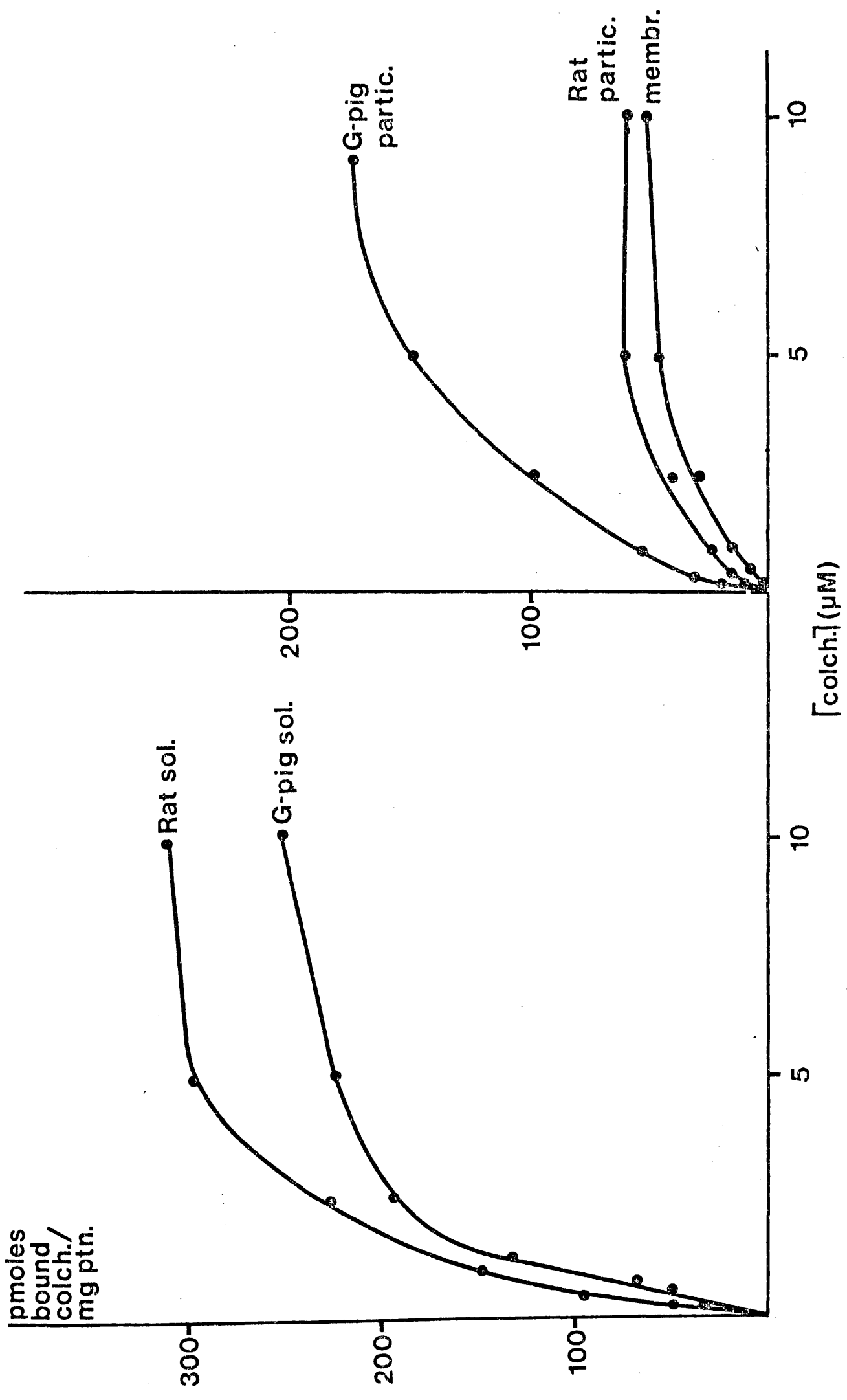


Figure 5.2

Reciprocal plot of colchicine-binding as a function
of colchicine concentration

Data presented in Figure 5.1 were used to obtain these graphs. For each of the preparations used, the value found for the binding constant, K , was within a very narrow range (2 to 3 μM) indicating that the binding proteins in the different preparations were very similar, and that there was a fairly high affinity of the protein for the substrate.

$(\text{pmoles } \times 10^{-3})^{-1}$

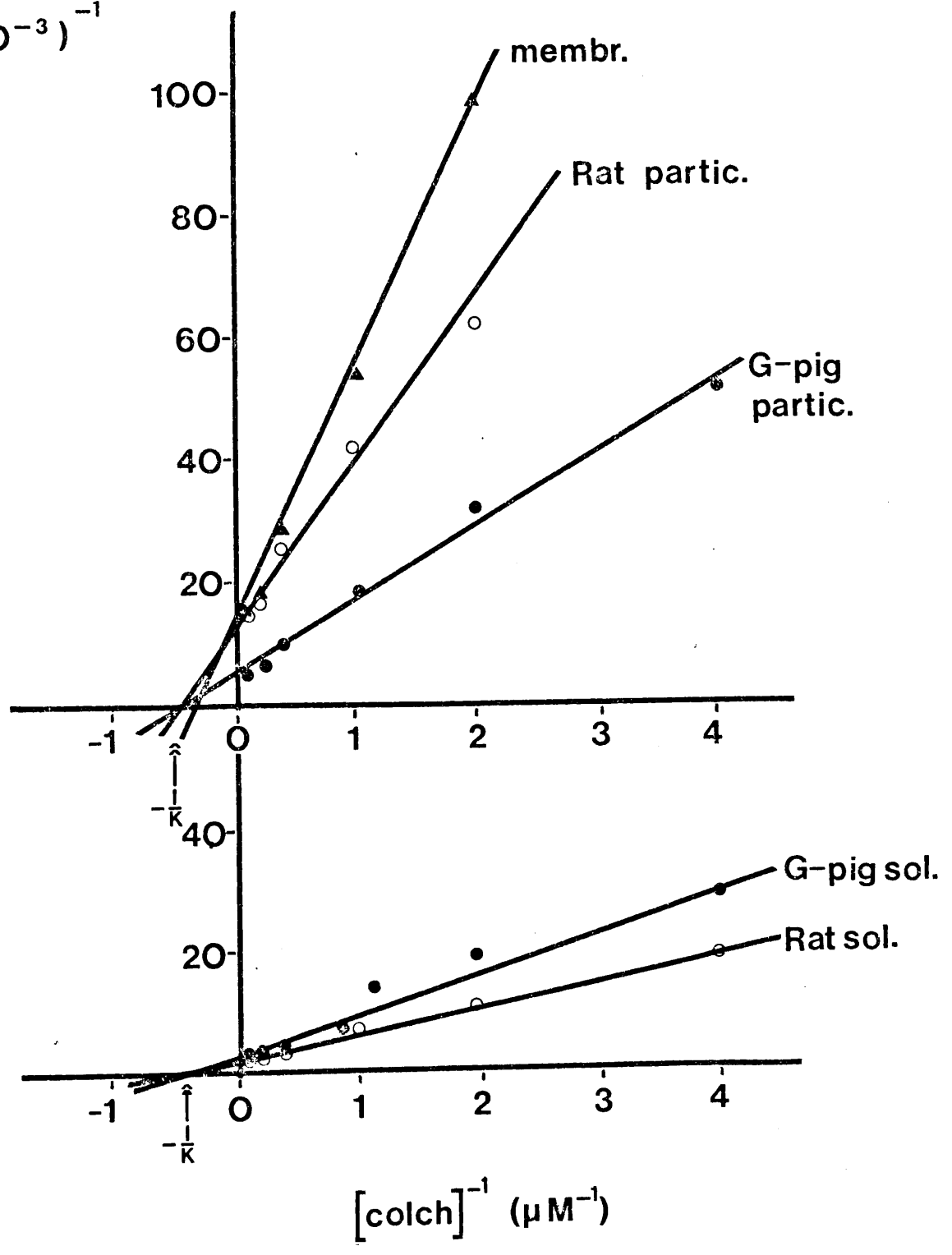


Figure 5.3

Colchicine-binding as a function of protein concentration

Soluble and particulate extracts of rat brain were prepared as described in Chapter 2. Binding mixtures were set up containing varied protein concentrations (as indicated) and $2.5 \mu\text{M}$ [^3H]-colchicine, and were incubated at 37° for 1.5h. They were then processed by the filter disc assay (see Chapter 2) to determine the amount of protein-bound radioactivity.

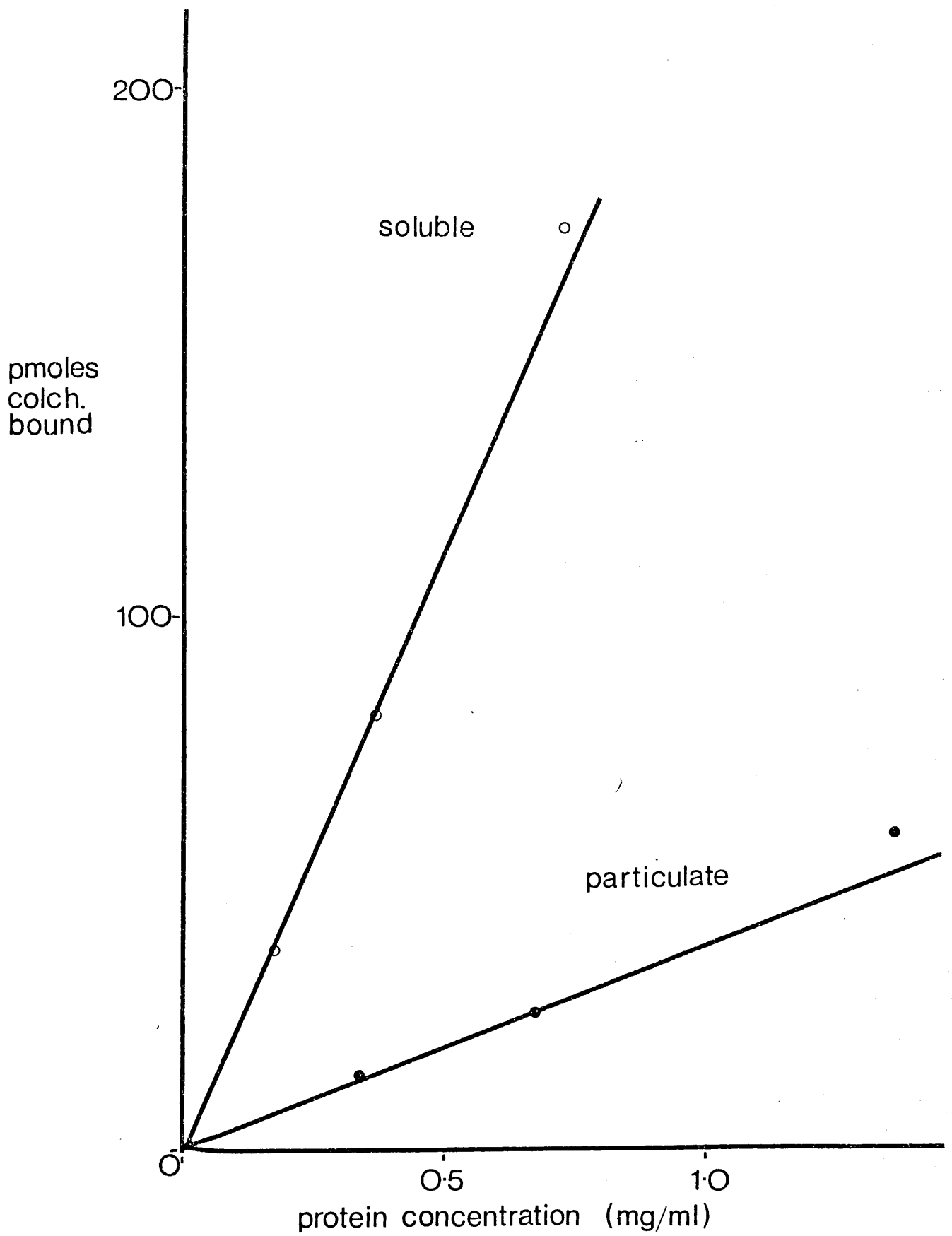
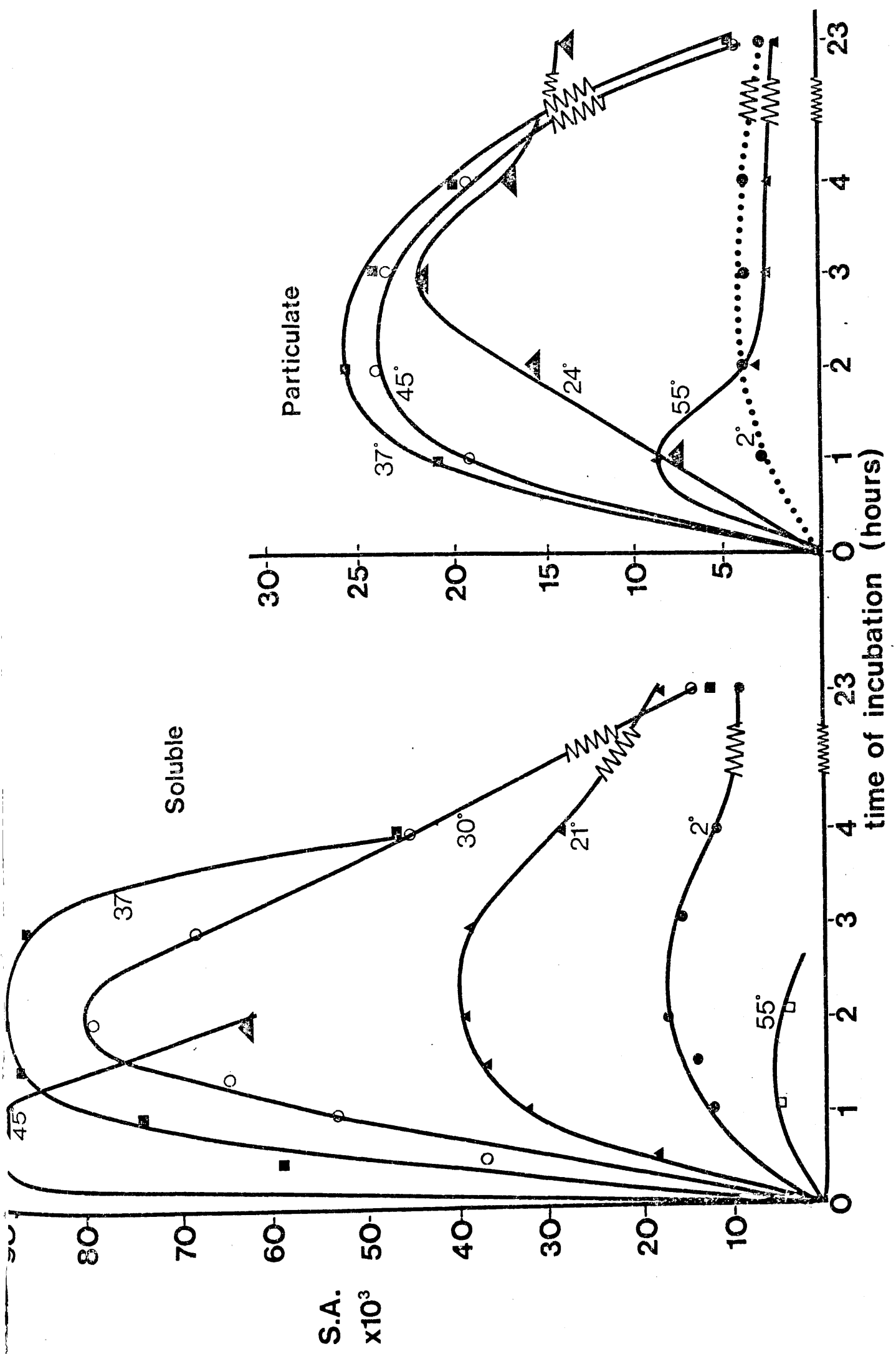


Figure 5.4

Colchicine-binding as a function of incubation
time and temperature

Rat brain soluble and particulate extracts were prepared as described in Chapter 2 and were assayed for CB-activity at various temperatures for different times (as indicated on the graphs). Aliquots were removed and processed by the filter disc assay to measure the amount of protein-bound radioactivity.

See also Figure 5.5.



23

4

3

2

1

0

10

20

30

40

50

60

70

80

90

time of incubation (hours)

Particulate

Soluble

S.A.
x10³

37°

45°

24°

55°

2°

37°

45°

30°

21°

2°

55°

23

4

3

2

1

0

10

20

30

40

50

60

70

80

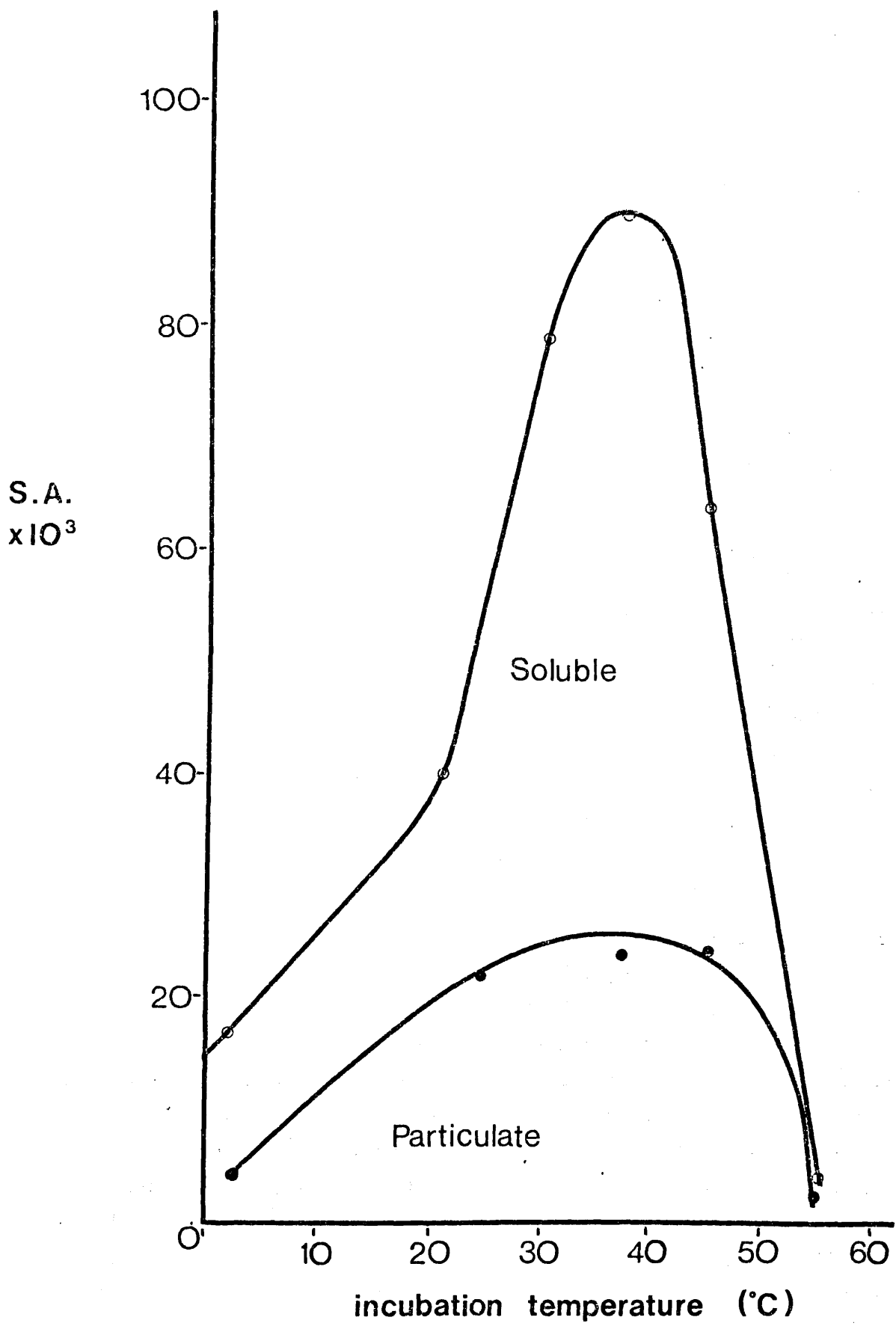
90

time of incubation (hours)

Figure 5.5

Colchicine-binding as a function of incubation temperature

The data shown in Figure 5.4 were used to obtain this graph. Soluble and particulate extracts of rat brain were assayed for CB-activity by incubation with 2.5 μM [^3H]-colchicine for 2h. at the various temperatures indicated. The samples were processed by the filter disc assay (see Chapter 2) to measure the amount of protein-bound radioactivity.



(d) Colchicine binding as a function of pH

Soluble and particulate preparations were assayed for CB-activity in 10mM sodium phosphate buffer, of varied pH, containing 10mM $MgCl_2$. Fig. 5.6 is a graphical representation of the results and shows that the optimal pH appears to be between pH 6.5 and pH 7.0 for both the preparations. The particulate CB-protein appears to be affected less by alterations in pH compared to the soluble preparation: at pH 5.0, CB-activity in both the samples is about 60% of the maximum, while at pH 8.0, CB-activity in the soluble preparation has been lowered to 70% of the maximum, but that in the particulate sample to only 85% of the maximum.

(e) Decay of CB-activity on storage at $-20^{\circ}C$

Rat brain homogenates were made in 0.32M sucrose containing 10mM phosphate- Mg^{2+} buffer and in 20mM phosphate-100mM glutamate buffer (see Materials and Methods). Soluble and particulate fractions were obtained and were assayed for CB-activity in the relevant buffer. The preparations were then stored at -20° for a given number of days, then were thawed and incubated as above. The freezing and thawing process itself was found to have little effect on CB-activity even after 5 successive freezings and thawings over a period of 2 hours. Therefore, the results shown in Fig. 5.7 reflect the decay in CB-activity due to the length of time stored frozen. The phosphate-glutamate preparations (in which CB-activity is reported to be stabilised against thermal inactivation at 37° : Wilson, 1970) have higher initial CB-activities, but after about 3 days storage these fall below the values seen in the phosphate- Mg^{2+} system. Table 5.1 shows the percentage loss of CB-activity in the samples.

Further experiments were carried out to test if the greater stability of CB-activity in the phosphate- Mg^{2+} buffered system was due to the presence of sucrose (which is normally present in this system, but is not normally present in the phosphate-glutamate buffered system). Homogenates were therefore also made using phosphate- Mg^{2+} buffer alone, and using phosphate-glutamate buffer containing 0.32M sucrose, and were spun to give soluble and particulate fractions. The CB-activity of the samples was determined on the day of preparation and after two days storage at -20° . It was found that addition of sucrose to the phosphate-glutamate samples had very little effect on the initial CB-activity or on the rate of decay of activity in this system. However, omission of sucrose from the phosphate- Mg^{2+} buffer, while giving similar initial CB-activities, resulted in a loss of 99% of the soluble CB-activity and

Figure 5.6

Colchicine-binding as a function of pH

Soluble and particulate extracts of rat brain were assayed for CB-activity in 10mM sodium phosphate of varied pH (as indicated) containing 10mM MgCl₂. The samples were processed by the filter disc assay (see Chapter 2) to measure the amount of protein-bound radioactivity.

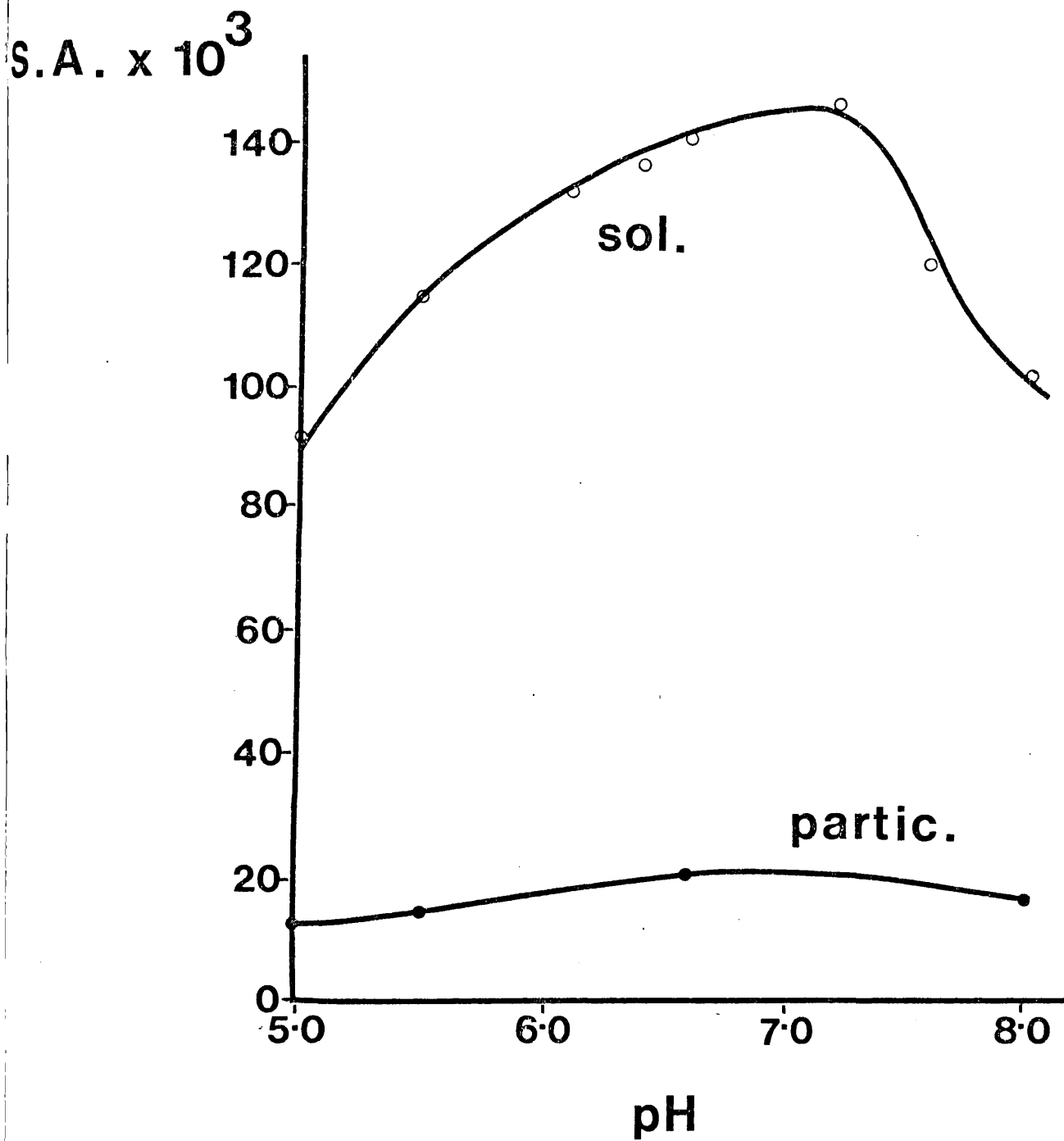
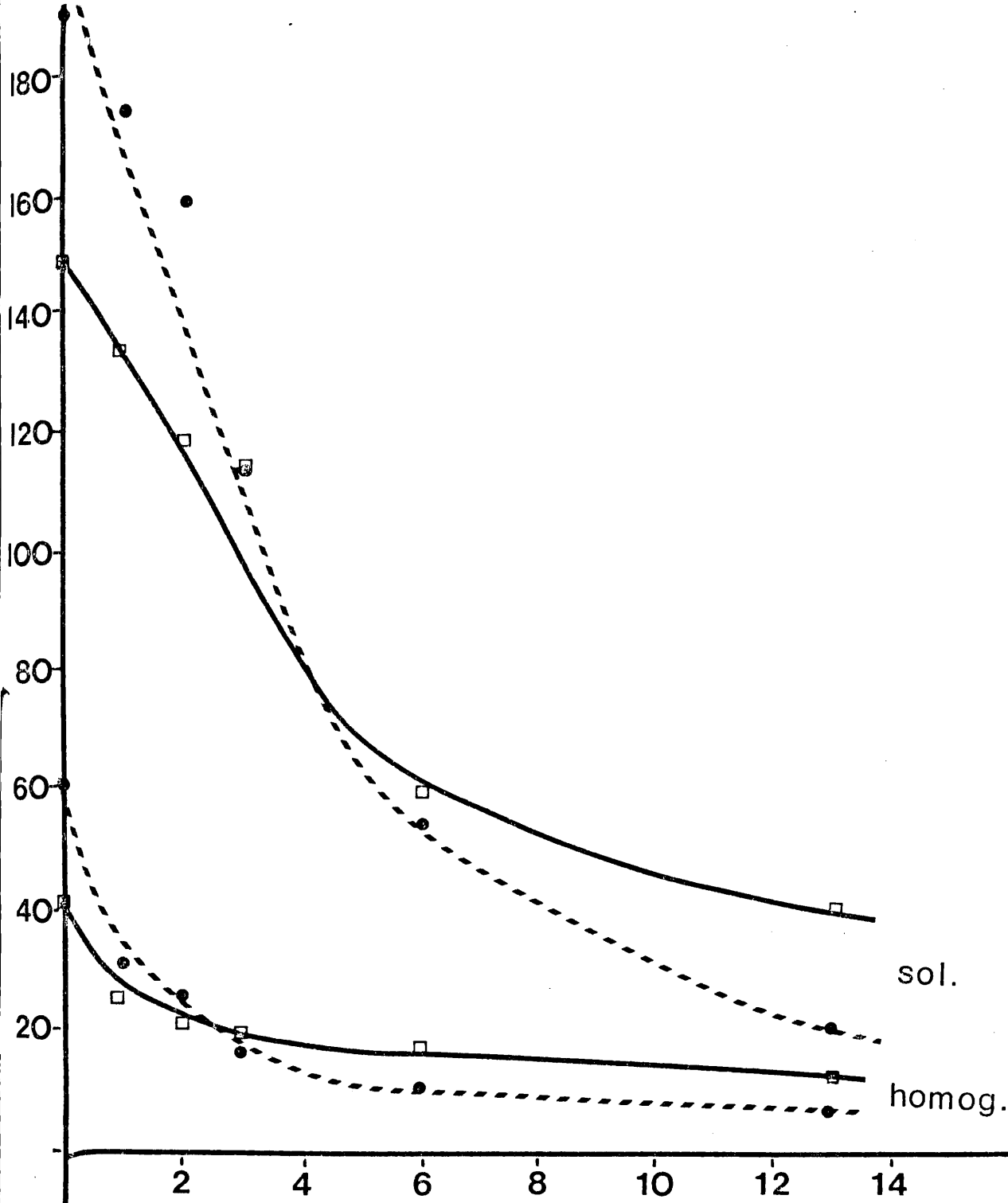


Figure 5.7

Decay of CB-activity at -20°

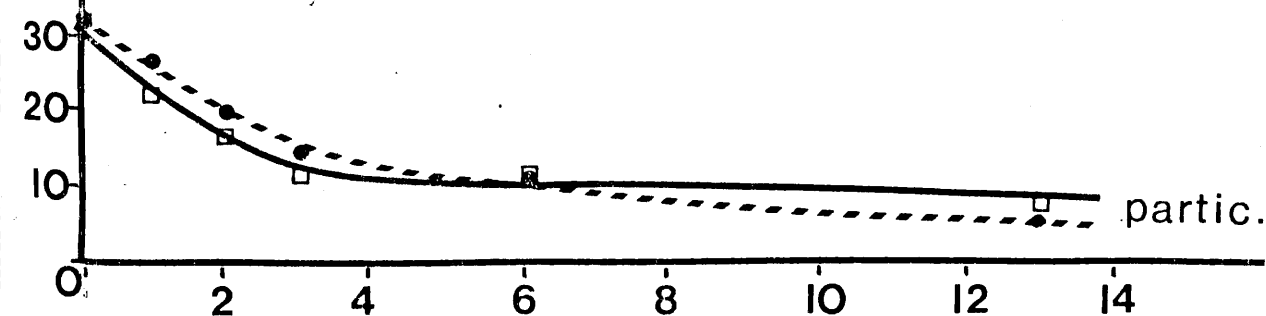
Rat brain was homogenised in 0.32M sucrose containing 10mM sodium phosphate buffer, pH 6.8, and 10mM $MgCl_2$ (—), or in 20mM sodium phosphate buffer, pH 7.0, containing 100mM sodium glutamate (- - -). Soluble and particulate extracts were obtained and were stored in the relevant buffer at -20° for the times indicated. They were then assayed for CB-activity. See also Table 5.1.

S.A. $\times 10^3$



sol.

homog.



partic.

DAYS STORED AT -20°C

Table 5.1

Decay of CB-activity at -20^o

The samples were prepared from rat brain homogenised in 0.32M sucrose containing 10mM phosphate buffer, pH 6.8, and 10mM MgCl₂ (P-Mg) and in 20mM phosphate buffer, pH 7.0, containing 100mM sodium glutamate (P-Gl). The extracts were assayed for CB-activity then were stored at -20^o for the times indicated before re-assay.

Values given are % of fresh CB-activity remaining at each time.

See also Figure 5.7.

Table 5.1

Decay of CB-activity at -20°

Sample	<u>Days frozen</u>				
	1	2	3	6	13
Homogenate (P-Mg)	61	51	46	35	23
Homogenate (P-Gl)	56	40	30	17	6
Particulate (P-Mg)	75	58	38	34	22
Particulate (P-Gl)	91	65	49	36	13
Soluble (P-Mg)	90	75	72	38	20
Soluble (P-Gl)	94	82	65	29	7

90% of the particulate CB-activity after storage for 2 days at -20° , as compared to losses of only 25-40% seen in sucrose-containing preparations (see Table 5.1). This observation clearly anticipated later studies which showed that colchicine-binding is protected by 0.5-1.0M sucrose (Frigon and Lee, 1972).

(f) Decay of CB-activity at 37°

Soluble and particulate protein preparations were incubated (protein concentration up to 1 mg/ml) at 37° for 0-3h prior to being assayed for CB-activity. The results obtained are given in Fig. 5.8, and show that CB-activity decreased as the length of preincubation (i.e. without colchicine) increased. It also appeared that the rate of decay of CB-activity was slightly faster in the soluble compared to the particulate preparation. If the results were plotted on a semi-logarithmic scale (Fig. 5.8 (b)), a linear decay rate was obtained for each of the preparations. From each of these rates, an estimate of the inactivation of CB-activity occurring during the 1.5h incubation with colchicine could be made by extrapolating the lines obtained. The zero preincubated samples were incubated with colchicine for 1.5h. Therefore if the lines were produced back to -1.5h this gave an estimate of the CB-activity of the samples at the beginning of the colchicine incubation, correcting for any inactivation occurring during the colchicine incubation itself. It was occasionally useful to compare decay rates of CB-activity by this method (see sections (j) and (k), and also Chapter 3, Fig. 5.9 in which soluble extracts from different areas of brain were studied) to determine whether or not initial CB-activity in different samples was the same.

(g) Effect of divalent cations (Mg^{2+} , Ca^{2+}) and different buffers on CB-activity

In preliminary experiments varied concentrations of these cations were used in the incubation mixtures to see if they had any effect on CB-activity during the binding reaction. The soluble preparation used was made from a homogenate prepared in 0.32M sucrose containing 10mM sodium phosphate buffer, pH 6.8. This was assayed for CB-activity in the presence of varied concentrations of Mg^{2+} or Ca^{2+} . Results obtained with varied concentrations of Mg^{2+} are given in Fig. 5.10, showing that the addition of up to 10mM Mg^{2+} stimulated CB-activity during the incubation with colchicine by up to 30%. Higher concentrations of Mg^{2+} resulted in no further stimulation of CB-activity. Similar results were obtained when Mg^{2+} was replaced by Ca^{2+} in the binding mixtures. In contrast, when up to 10mM Mg^{2+} or Ca^{2+} was added to incubation mixtures containing particulate preparations, no stimulation of CB-activity was observed.

Figure 5.8

Decay of CB-activity at 37^o

Soluble and particulate extracts of rat brain were incubated (protein concentration approximately 1mg/ml) in 10mM phosphate-Mg²⁺ buffer at 37^o for the times indicated prior to the addition of 2.5 μ M [³H]-colchicine. The samples were then re-incubated at 37^o for a further 1.5h. and were processed, in triplicate, by the filter disc assay (see Chapter 2) to determine the amount of protein-bound radioactivity. The results in (b) are extrapolated to zero time of incubation to correct for loss in CB-activity during the whole incubation procedure. The results presented are the means from 3 separate experiments.

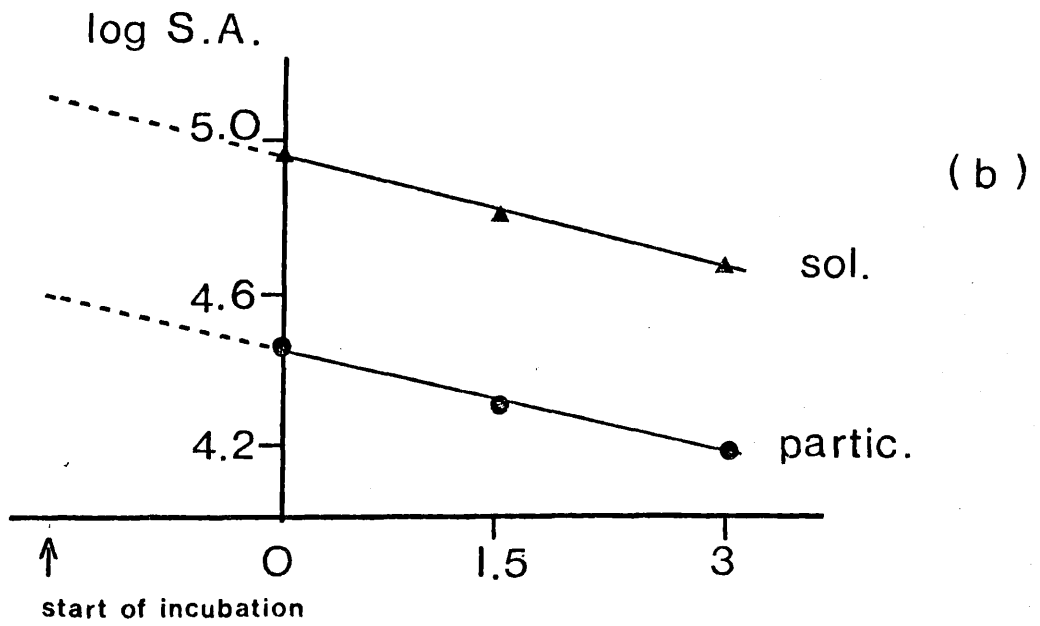
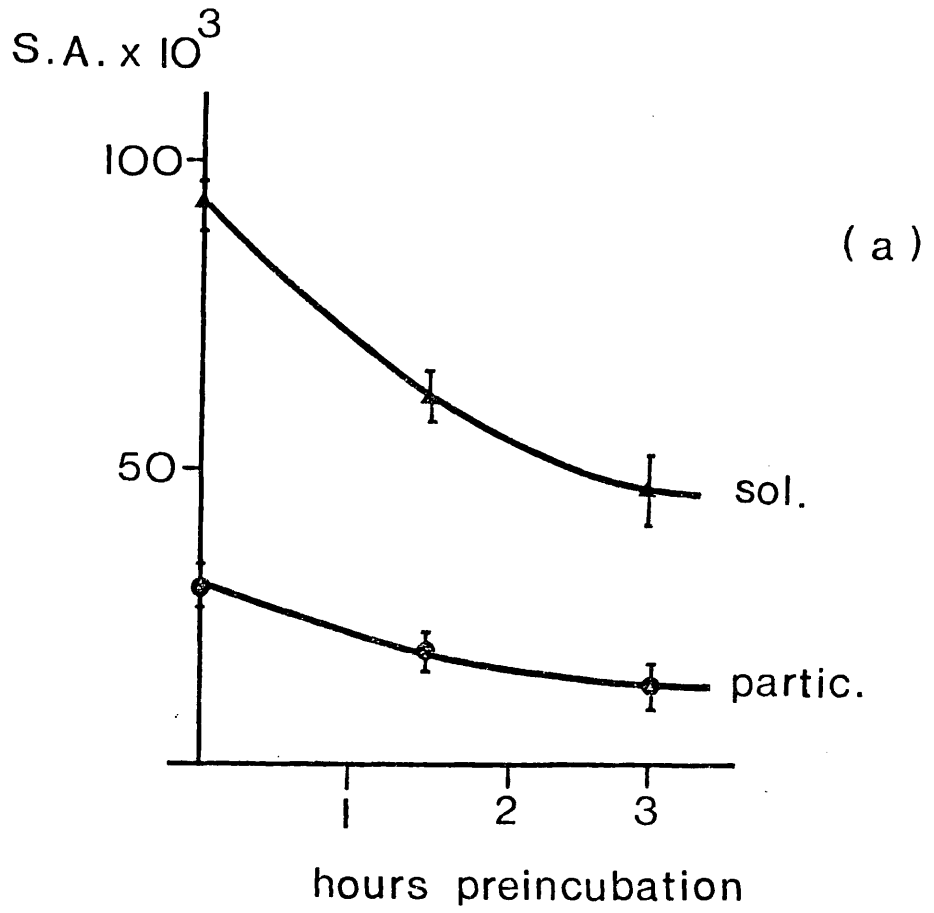


Figure 5.9

Decay of CB-activity at 37⁰ in soluble extracts
from various anatomical locations of rat brain

Soluble extracts of rat brain were prepared from the following anatomical regions of brain: cortex (C), thalamus/hypothalamus (T), cerebellum (Ce) and brain stem (S). Aliquots were pre-incubated at 37⁰ for the times shown prior to addition of 2.5 μM [³H]-colchicine and further incubation for 1.5h. Samples were then processed by the filter disc assay (see Chapter 2) to determine the amount of protein-bound radioactivity. The results are extrapolated to estimate the initial CB-activity present in the samples at the start of the incubation procedure. These results were used to obtain Figure 3.5 and are the means from 2 separate experiments.

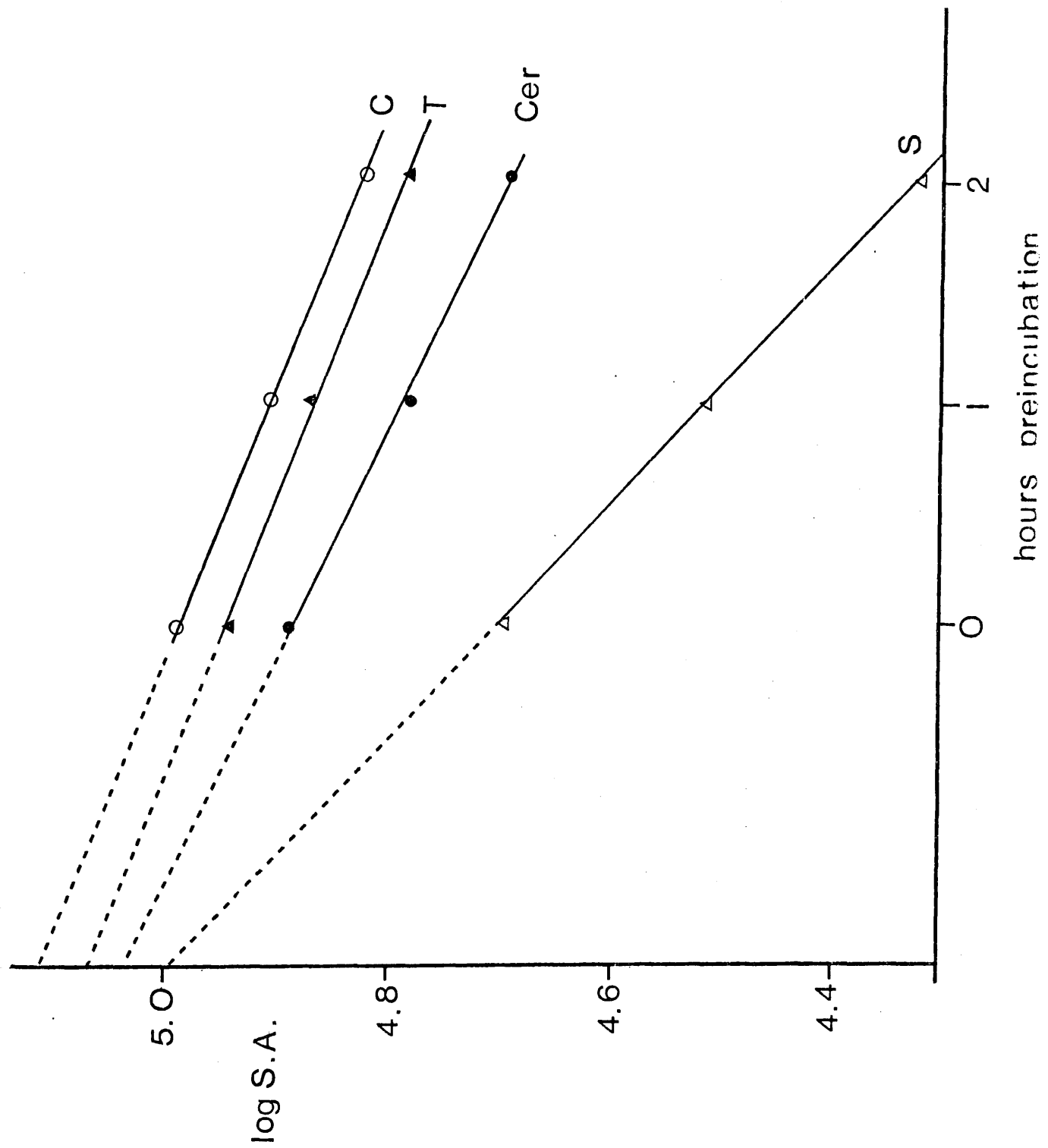
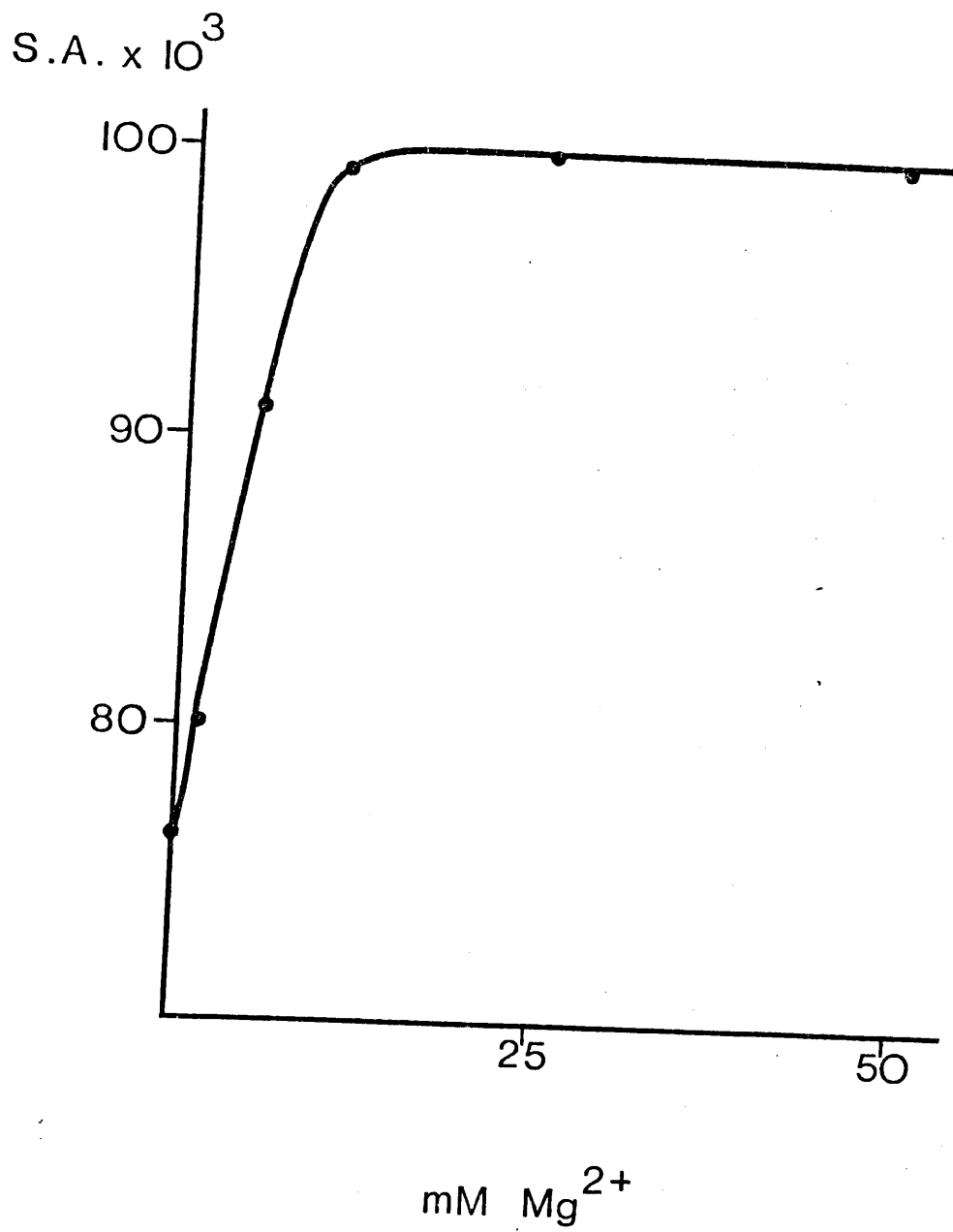


Figure 5.10

Colchicine-binding as a function of Mg^{2+} concentration

A soluble extract was prepared from rat brain using 0.32M sucrose containing 10mM sodium phosphate buffer, pH 6.8. Binding mixtures were set up containing approximately 1mg/ml of protein, $2.5 \mu M$ [3H]-colchicine, and varied concentrations of $MgCl_2$, as indicated. The mixtures were incubated at 37° for 1.5h., then were processed by the filter disc method (see Chapter 2) to determine the amount of protein-bound radioactivity.



It was possible that even higher stimulation of CB-activity could be obtained if Mg^{2+} or Ca^{2+} were also included in the homogenising medium. Brain homogenates were therefore prepared in 0.32M sucrose containing varied concentrations of phosphate buffer and Mg^{2+} . Soluble and particulate preparations were obtained from them, and these were assayed for CB-activity using the relevant incubation buffer. Results are given in Table 5.2, showing again that inclusion of 1-10mM Mg^{2+} resulted in a stimulation of CB-activity. However, addition of 50mM Mg^{2+} caused a decrease in CB-activity compared to the control samples which contained no Mg^{2+} . This could possibly be due to aggregation of CB-protein into aggregates which bind colchicine only poorly (Weisenberg and Timasheff, 1970). It was also observed that as the buffer concentration increased up to 50mM, there was an accompanying increase in measured CB-activity. These changes were found not to be due to differences in protein distribution in the different samples, and CB-activity was altered to a similar extent in the fractions in all the conditions tested.

It therefore seems that these effects could be due to increased ionic strength; this would confirm the results of Wilson (1970) who also showed that addition of sodium chloride or sodium glutamate stabilised CB-activity by altering the half-time of the inactivation process, maximal effects occurring at a salt concentration of 200mM.

(h) Effect of thiol reagents on CB-activity

A soluble preparation was incubated with [3H]-colchicine and various thiol reagents to find if these would cause a stimulation of CB-activity. The reagents used were: β -mercaptoethanol, dithiothreitol and dithiodipropanol, each at a final concentration of 5mM. It was observed, however, that no stimulation in CB-activity was obtained.

(i) Effect of GTP on CB-activity

In preliminary experiments, the effects of incorporating 10^{-4} M GTP into the homogenising and incubation buffers were investigated. Homogenates were prepared in 0.32M sucrose containing 1mM phosphate buffer and 1mM $MgCl_2$, with or without added GTP. The homogenates were centrifuged to obtain soluble and particulate fractions. These were assayed for CB-activity in the presence or absence of GTP. It was found that addition of GTP had very little effect on CB-activity in the homogenate or particulate fraction compared to the control samples. However, in the soluble fraction, the presence of GTP resulted in almost double the CB-activity measured in the control sample. This difference was found not to be due to different distributions of CB-activity or protein in the control and GTP samples.

Table 5.2

Effects of varied homogenisation and incubation buffers on soluble and particulate CB-activity

Brain homogenates were made in 0.32M sucrose containing each of the buffers listed. These were centrifuged for 10^5 g-h. to obtain soluble and particulate preparations, and the extracts were separately incubated (protein concentration approximately 1mg/ml) in the respective buffer (no sucrose) with $2.5 \mu\text{M}$ [^3H]-colchicine for 1.5h. at 37° . Samples were processed by the filter disc method (see Chapter 2) to determine the amount of protein-bound radioactivity.

Table 5.2

Effects of varied homogenisation and incubation buffers on soluble and particulate CB-activity

<u>Buffer</u>	<u>Soluble S.A. ($\times 10^3$)</u>	<u>Particulate S.A. ($\times 10^3$)</u>
1mM phosphate (pH 6.8)	40	20
1mM phosphate (pH 6.8) + 1mM Mg ²⁺	55	30
1mM phosphate (pH 6.8) + 10mM Mg ²⁺	75	36
<hr/>		
10mM phosphate (pH 6.8)	78	33
10mM phosphate (pH 6.8) + 1mM Mg ²⁺	100	36
10mM phosphate (pH 6.8) + 10mM Mg ²⁺	103	45
<hr/>		
50mM phosphate (pH 6.8)	153	85
50mM phosphate (pH 6.8) + 50mM Mg ²⁺	92	67
<hr/>		
100mM phosphate (pH 6.8)	133	92

The effect of various concentrations of GTP on CB-activity in a soluble preparation were next investigated. A homogenate was made in 0.32M sucrose containing 10mM phosphate buffer and 10mM $MgCl_2$, and this was centrifuged to produce the soluble extract. This was assayed for CB-activity in the presence of various concentrations of GTP. The results obtained are given in Fig. 5.11, showing that almost maximal stimulation of CB-activity in the soluble fraction was obtained using a concentration of 1mM GTP. It is also of interest that 0.1mM GTP gave a much smaller stimulation of CB-activity when 10mM phosphate- Mg^{2+} buffer was used compared to that found in the preliminary experiments in which the buffer used was 1mM phosphate- Mg^{2+} . It is possible that the higher concentration of buffer and Mg^{2+} were also contributing to the stimulation of CB-activity (see section (g)), so the effect of added GTP did not appear so great.

It was of interest to discover if this stimulation of CB-activity remained as CB-activity decayed in the incubation at 37° (see section (f)). Soluble and particulate extracts were obtained from a homogenate prepared in 0.32M sucrose containing 10mM phosphate- Mg^{2+} buffer. The extracts were then incubated (protein concentration approximately 1 mg/ml) in the presence and absence of added 1mM GTP for various times at 37°. They were then assayed for CB-activity and the results obtained are given in Fig. 5.12, showing that the initial stimulation of CB-activity obtained was retained in both the samples; in fact, in the samples containing no GTP, CB-activity decayed at a faster rate. In addition, it can be seen that CB-activity at the start of the incubation was greater in the samples containing GTP than in the control samples, indicating that GTP did also stimulate the initial CB-activity.

(j) Effects of Vinca alkaloids on CB-activity

The Vinca alkaloid vinblastine at a concentration of 1mM is known to precipitate CB-protein from certain types of cells (Marantz et al (1969); Marantz and Shelanski (1970); Olmsted et al (1970)) in the presence of magnesium ions. Vincristine has also been used to stabilise CB-activity in extracts of brain (Wilson, 1970). Further effects of these alkaloids on CB-activity were therefore investigated.

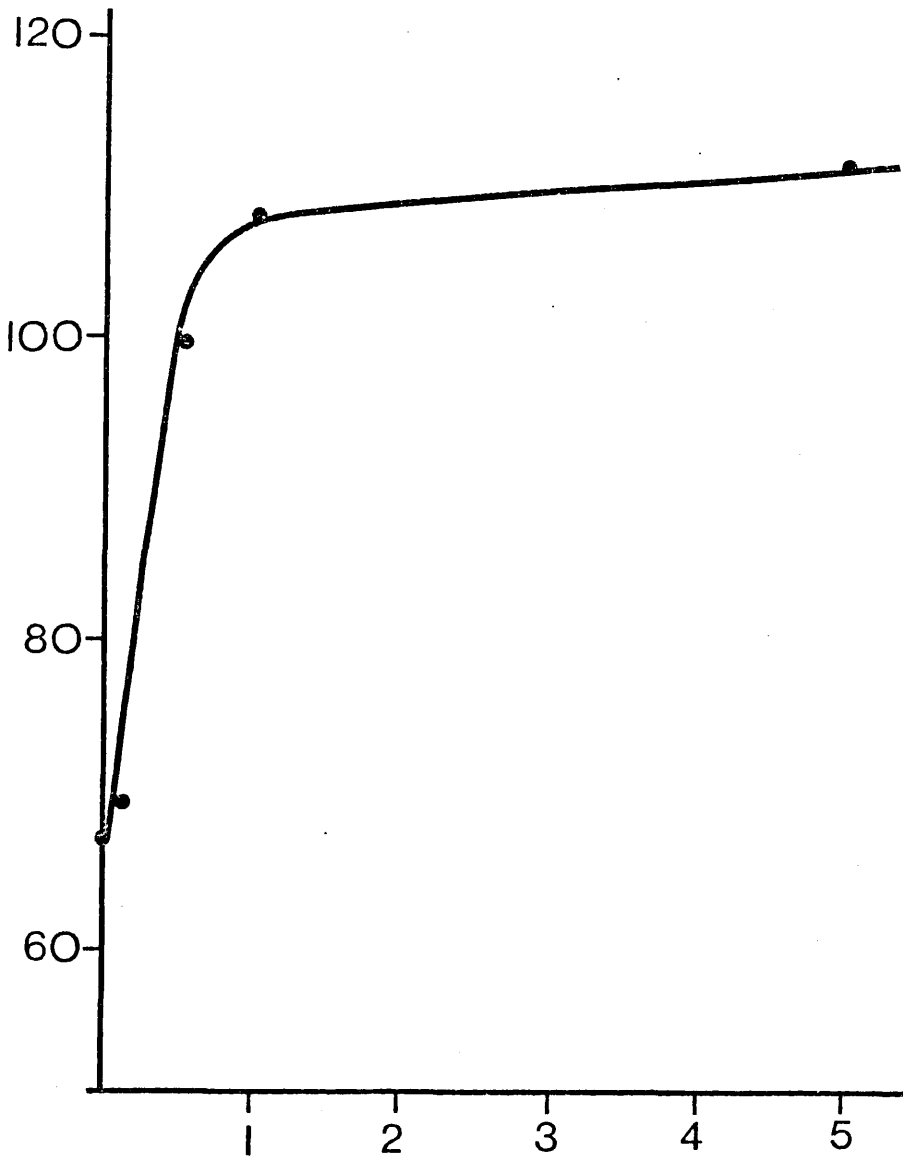
In a preliminary experiment a relatively low concentration of vincristine (50 μ M) was included in the colchicine incubation to test its effect on CB-activity. A soluble extract of brain was used, obtained from a homogenate made in 0.32M sucrose containing 1mM phosphate buffer and 1mM $MgCl_2$. This was assayed for CB-activity in the presence and absence of added 50 μ M vincristine. It was found that the measured

Figure 5.11

Colchicine-binding as a function of GTP concentration

A rat brain soluble extract was incubated (protein concentration approximately 1mg/ml) with 2.5 μM [^3H]-colchicine and varied GTP concentrations (as shown) for 1.5h. at 37 $^{\circ}$. The samples were then processed by the filter disc assay (see Chapter 2) to determine the amount of protein-bound radioactivity.

S.A. $\times 10^3$

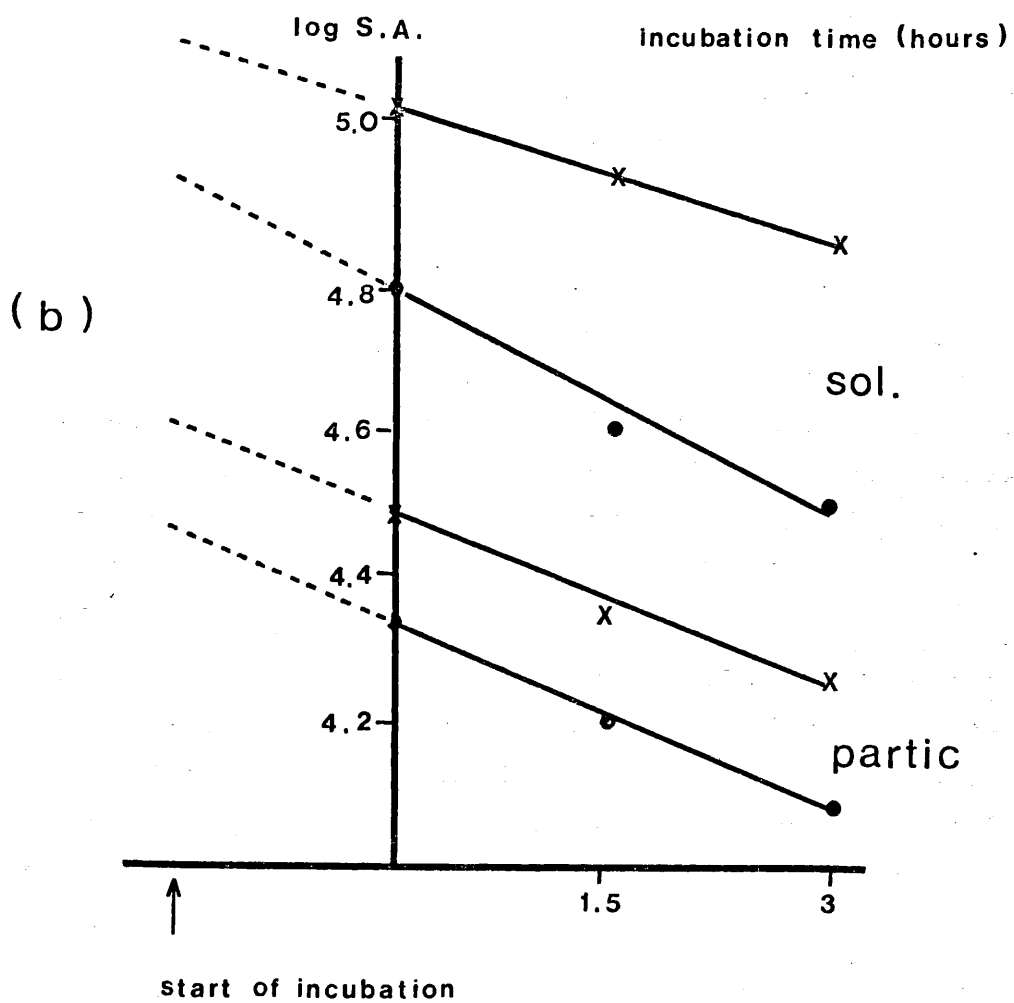
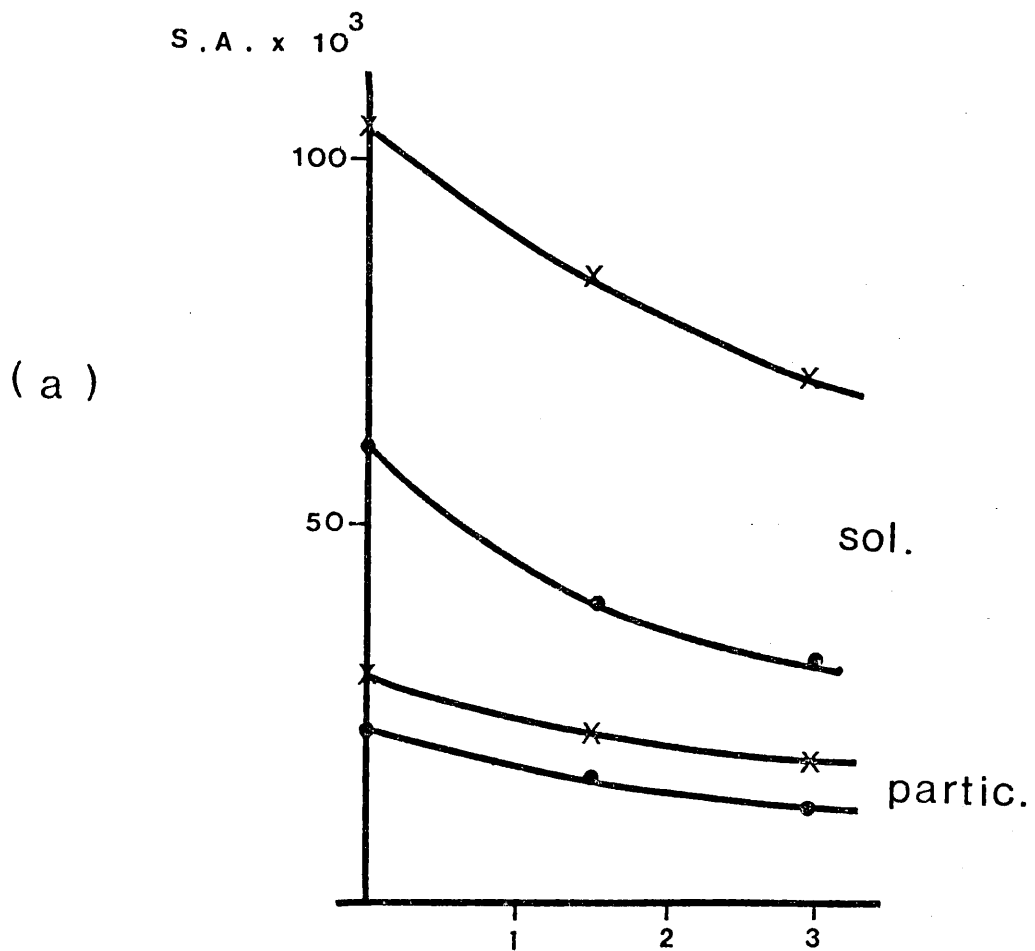


mM GTP

Figure 5.12

Decay of CB-activity at 37^o in the presence and absence of GTP

Soluble and particulate extracts of rat brain were incubated (protein concentration approximately 1mg/ml) with (X) or without (•) added GTP (1mM), at 37^o for the times indicated. 2.5 μ M [³H]-colchicine was then added and the samples were reincubated for a further 1.5h. Triplicate samples were then processed by the filter disc assay (see Chapter 2) to determine the amount of protein-bound radioactivity. The results given are the means from 2 separate experiments. Results in (b) are extrapolated to zero time of incubation to correct for losses in CB-activity occurring during the incubation procedure.



CB-activity in the sample containing vincristine was about 30% higher than that measured in the control sample.

The effect of incorporating vincristine in the homogenising medium was next investigated. Homogenates were made in 0.32M sucrose containing 10mM phosphate buffer and 10mM $MgCl_2$, with and without added vincristine at a concentration of 50 μM . These were centrifuged and the soluble and particulate fractions obtained. Each of the samples was then assayed for CB-activity and it was found that CB-activity in the homogenate was almost doubled by addition of vincristine; however, CB-activity had undergone a redistribution in the subfractions. The control soluble fraction contained about half the recovered CB-activity, but in the vincristine soluble fraction this had been reduced to only about 5% of the total recovered. Protein distribution in the two sets of samples was not significantly different, and over 80% of each homogenate CB-activity and total protein had been recovered.

It was apparent, therefore, that CB-protein had been redistributed from the soluble to the particulate fraction. If this were the case, it should be possible to cause a similar redistribution by adding vincristine to a soluble preparation and recentrifuging, to precipitate the CB-protein. The dependence of this result on the presence of magnesium ions was also tested in this experiment. A soluble preparation was obtained from a homogenate made in 0.32M sucrose containing 10mM phosphate buffer.

Aliquots of this were treated as follows:

- 1 no further additions
- 2 10mM $MgCl_2$ added
- 3 50 μM vincristine added
- 4 10mM $MgCl_2$ and 50 μM vincristine added.

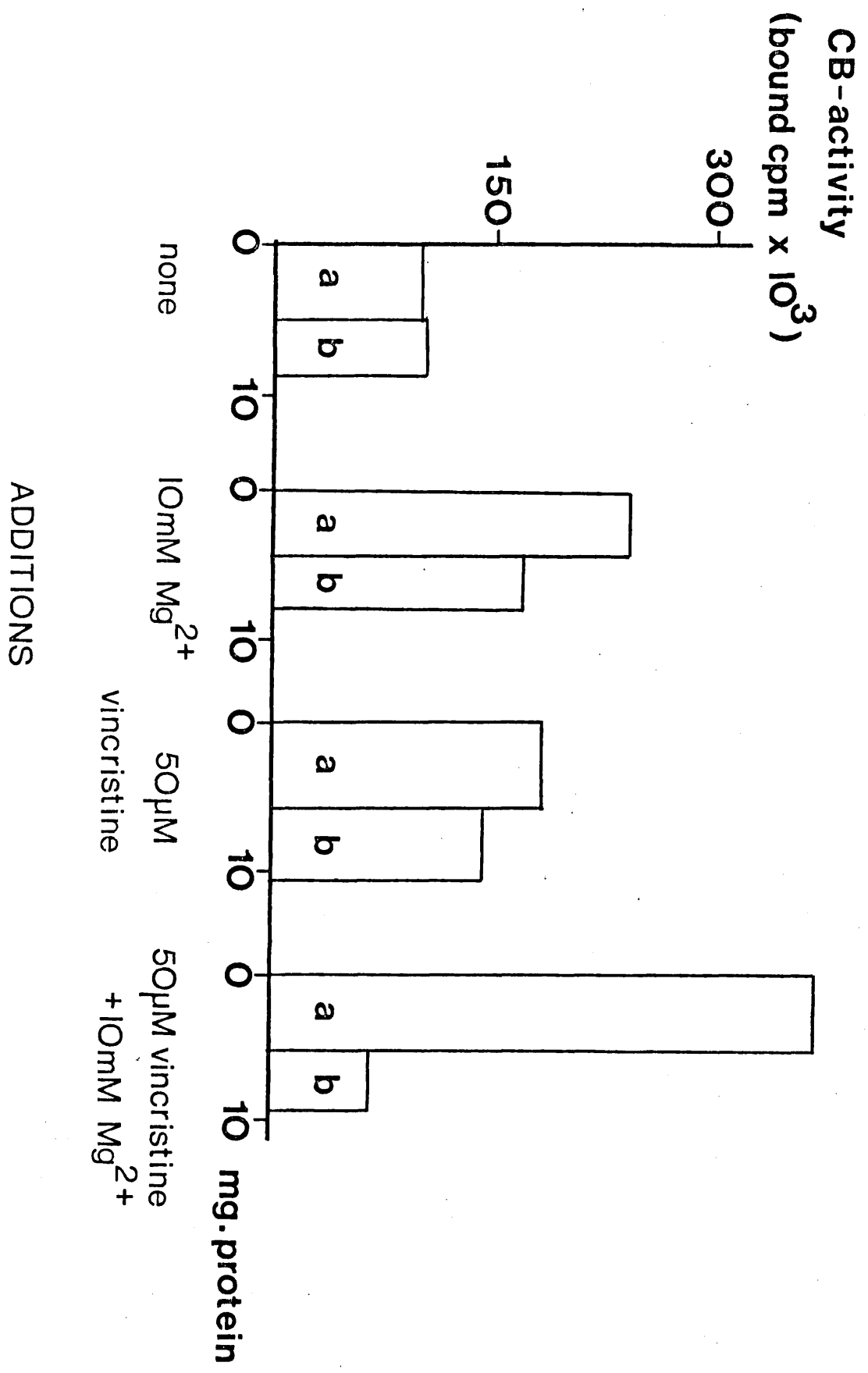
The samples were then all recentrifuged for 10⁵g-h. All the soluble samples were assayed for CB-activity and the results obtained are given in Fig. 5.13. It can be seen that the majority of the CB-activity had been removed from the sample containing both 10mM $MgCl_2$ and 50 μM vincristine after recentrifugation. The other samples did not show a significant change.

The samples containing both 10mM $MgCl_2$ and 50 μM vincristine (including the very small precipitate) were subjected to SDS-polyacrylamide gel electrophoresis (as described in Chapter 2) in order to analyse which molecular weight proteins were concerned with the observed redistribution of CB-activity. Purified CB-protein (see Chapter 6) was used as a standard.

Figure 5.13

Precipitation of CB-protein from a soluble extract
of brain using vincristine

A soluble extract of rat brain was prepared in 0.32M sucrose containing 10mM phosphate buffer, pH 6.8. Samples of this were treated with the additions shown and all the samples were recentrifuged for 10^5 g-h. Aliquots before recentrifuging (a) and after recentrifuging (b) were assayed for CB-activity.



The results of the electrophoresis obtained are shown in Fig. 5.14, from which it can be seen that the vincristine-precipitated material contained a major component which migrated in the same manner as the purified preparation. It also appears that in the soluble fraction obtained after recentrifuging, this component is present in a much lower concentration compared to that in the original soluble sample. From this evidence, therefore, it seems that the low concentration of vincristine used, in the presence of magnesium ions, will selectively precipitate CB-protein in the cold from extracts containing it. Vinblastine has also been used, in similar experiments, with the same results. In further experiments it was found that the concentration of alkaloid needed to achieve this effect could be as low as $5 \mu\text{M}$, but the accompanying concentration of magnesium ions had to be greater than 1mM . If higher concentrations of the alkaloid were used (greater than 0.5mM) and the samples were incubated at 37° for at least 10 minutes, the precipitation could be observed as the solution became turbid. The precipitate was then collected by centrifuging for 20 minutes at $20,000g$. Precipitation was found to occur in all the soluble extracts tested in which the protein concentration varied between 0.2 to 10.0 mg/ml . Similarly, incubation of brain extracts with high concentrations of vinblastine has been shown to cause selective precipitation of tubulin (Marantz and Shelanski, 1970).

It was possible that the precipitation of CB-protein from brain extracts could be due to an interaction between vincristine and other factors present in the extracts. This idea was tested using purified CB-protein (see Chapter 6) which had been prepared in 10mM phosphate buffer containing 10mM MgCl_2 . Vincristine (concentration $50 \mu\text{M}$) was added to the sample, which was then centrifuged for $10^5 g$ -h. Aliquots of the preparation before and after centrifuging were assayed for CB-activity and it was found that over 90% of the CB-activity and about 60% of the protein had been precipitated from the sample after recentrifugation. In a control sample (which contained no vincristine) the CB-activity decreased by only about 10% after recentrifuging. It appeared, therefore, that other constituents present in brain extracts were not required for the vincristine-induced precipitation of CB-protein.

Experiments were next conducted to investigate the effects of varied concentrations of vincristine on CB-activity. Soluble and particulate extracts were prepared from a homogenate made in 0.32M sucrose containing 10mM phosphate buffer and 10mM MgCl_2 and the extracts were assayed for colchicine-binding in the presence of different concentrations of vincristine.

Figure 5.14

Electrophoresis of vincristine-precipitated samples

To a soluble extract of rat brain prepared in P-Mg-sucrose buffer (photograph 1) was added vincristine (at a final concentration of 50 μM), and the sample was centrifuged for 10⁵ g-h. Some protein precipitated (see photograph 3), leaving the soluble fraction depleted in one main band (arrowed) (see photograph 2). This corresponded to a molecular weight of approximately 60,000. This precipitation was shown, from CB-assays on the samples, to be accompanied by a selective precipitation of CB-activity from the original supernatant (see text). Electrophoresis was performed as described in Chapter 2, using phosphate-SDS buffer and loading each gel with approximately 100 ug of protein. The gels were fixed and stained with Coomassie brilliant blue.

The results, given in Fig. 5.15, show that almost maximal stimulation of CB-activity was obtained using 100 μ M vincristine, and that CB-activity in these samples was approximately double that in the samples which contained no vincristine.

It was possible that the stimulation of CB-activity by vincristine was partly due to protection of the CB-protein from the inactivation process occurring during the incubation with colchicine. Experiments were therefore carried out to test this possibility. Soluble and particulate extracts were prepared from a homogenate made in 0.32M sucrose containing 10mM phosphate buffer and 10mM $MgCl_2$. Vincristine was added to half of each sample at a final concentration of 0.5mM, and the control and vincristine-containing samples were incubated for various times at 37^o. They were then assayed for CB-activity. The results, given in Fig. 5.16, show that CB-activity decayed at a slower rate in the samples containing vincristine than in the control samples. Also, the extrapolated initial values for colchicine-binding were higher in the vincristine-containing samples (see Fig. 5.16 (b)).

(k) Solubilisation of membrane-bound CB-protein

In preliminary experiments, attempts to solubilise particulate-bound CB-activity were unsuccessful. The crude particulate fraction obtained from brain homogenates was resuspended in homogenising buffer and was recentrifuged. CB-activity in the samples before and after recentrifuging was determined, and it was found that the particulate fraction remaining after recentrifugation still contained about 90% of the original crude particulate CB-activity.

Non-ionic detergents (e.g. Triton X-100) are commonly used to solubilise membrane-bound proteins, so a homogenising medium was prepared which contained 2% Triton X-100. Soluble and particulate extracts obtained from such derived homogenates were assayed for CB-activity. The CB-activity of the particulate fraction from Triton-containing homogenates was about 80% that of controls, although it contained only about 60% of the protein present in control particulate preparations. This concentration of Triton had very little effect on CB-activity during the incubation at 37^o, indicating that CB-protein had not been selectively solubilised. On the contrary, it appeared that enrichment of particulate CB-activity had occurred; this could, however, be attributed to the smaller percentage of protein pelleted.

Sonication is a technique sometimes used to solubilise membrane-bound proteins. A brain homogenate was therefore prepared in 0.32M sucrose

Figure 5.15

Colchicine-binding as a function of vincristine concentration

To soluble and particulate extracts of rat brain were added varying concentrations of vincristine (as indicated); the samples were then assayed for CB-activity.

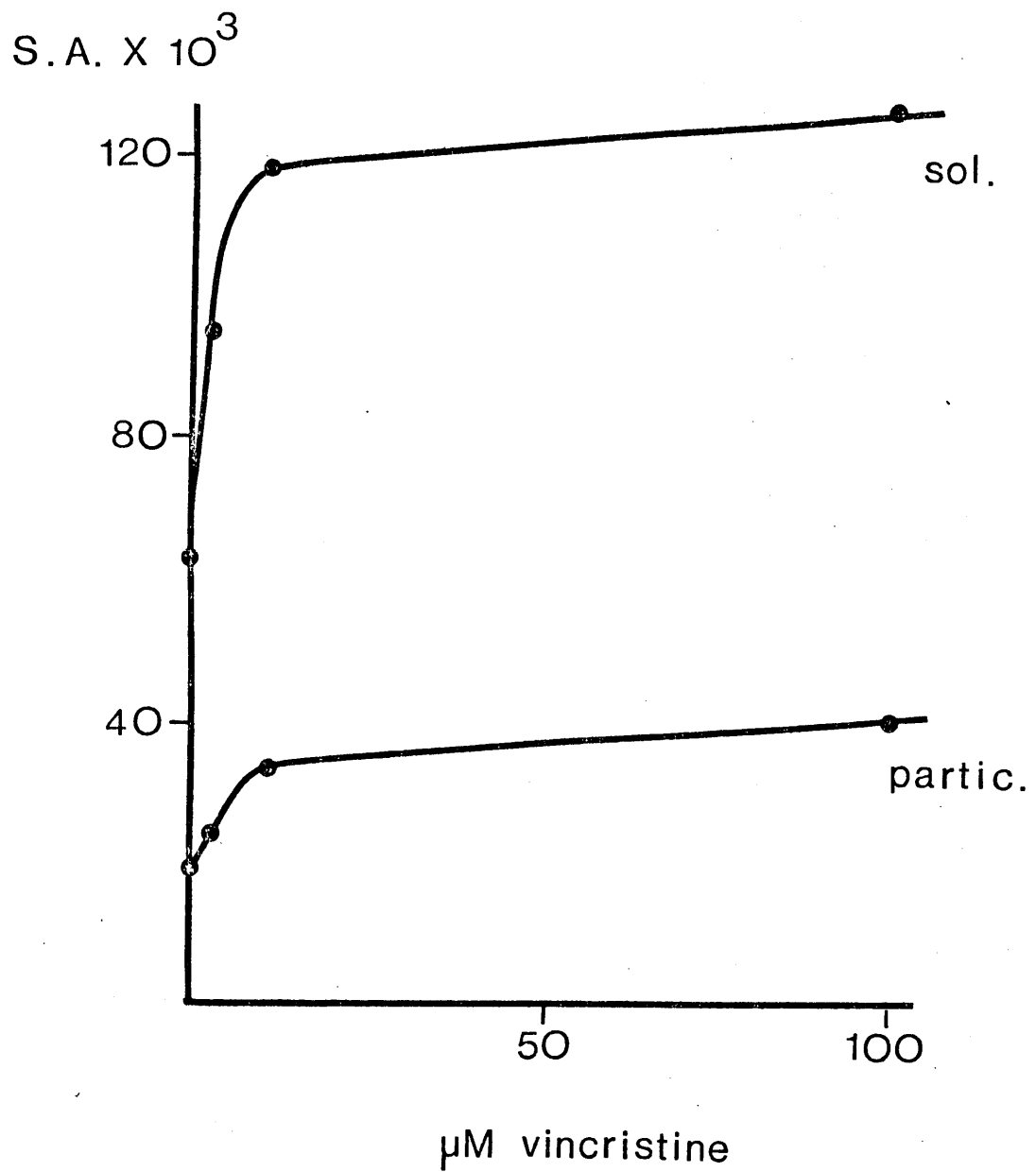
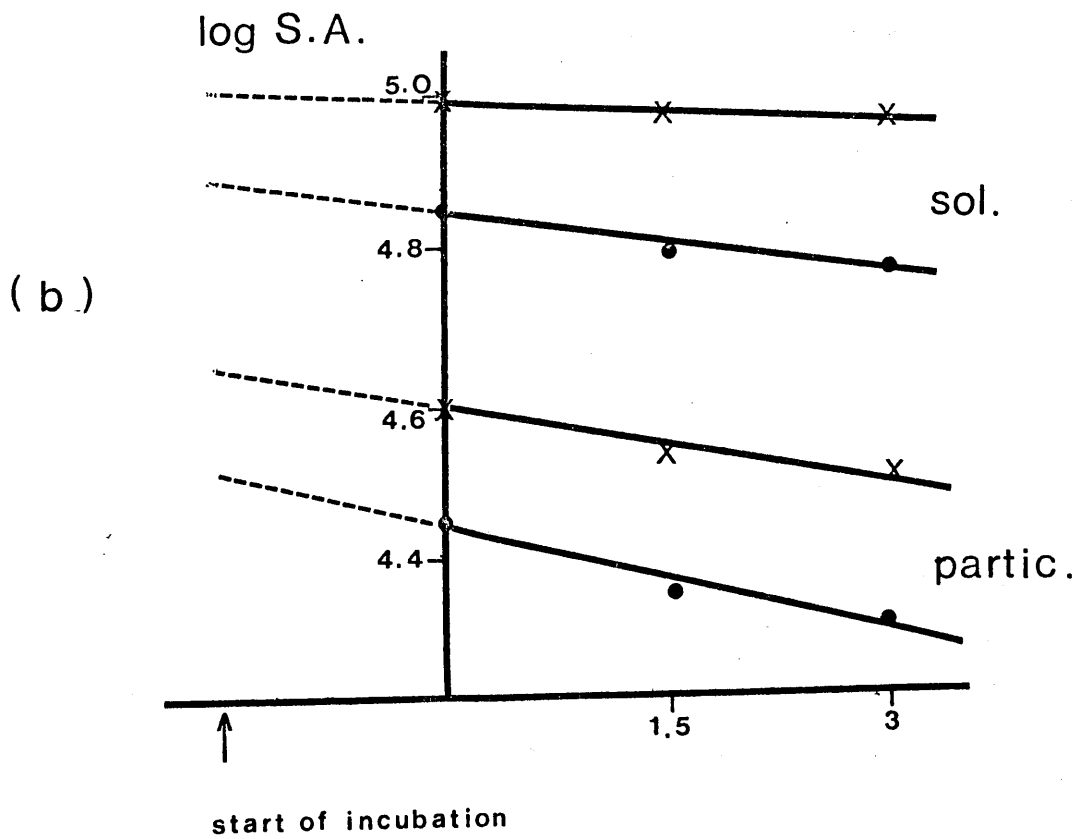
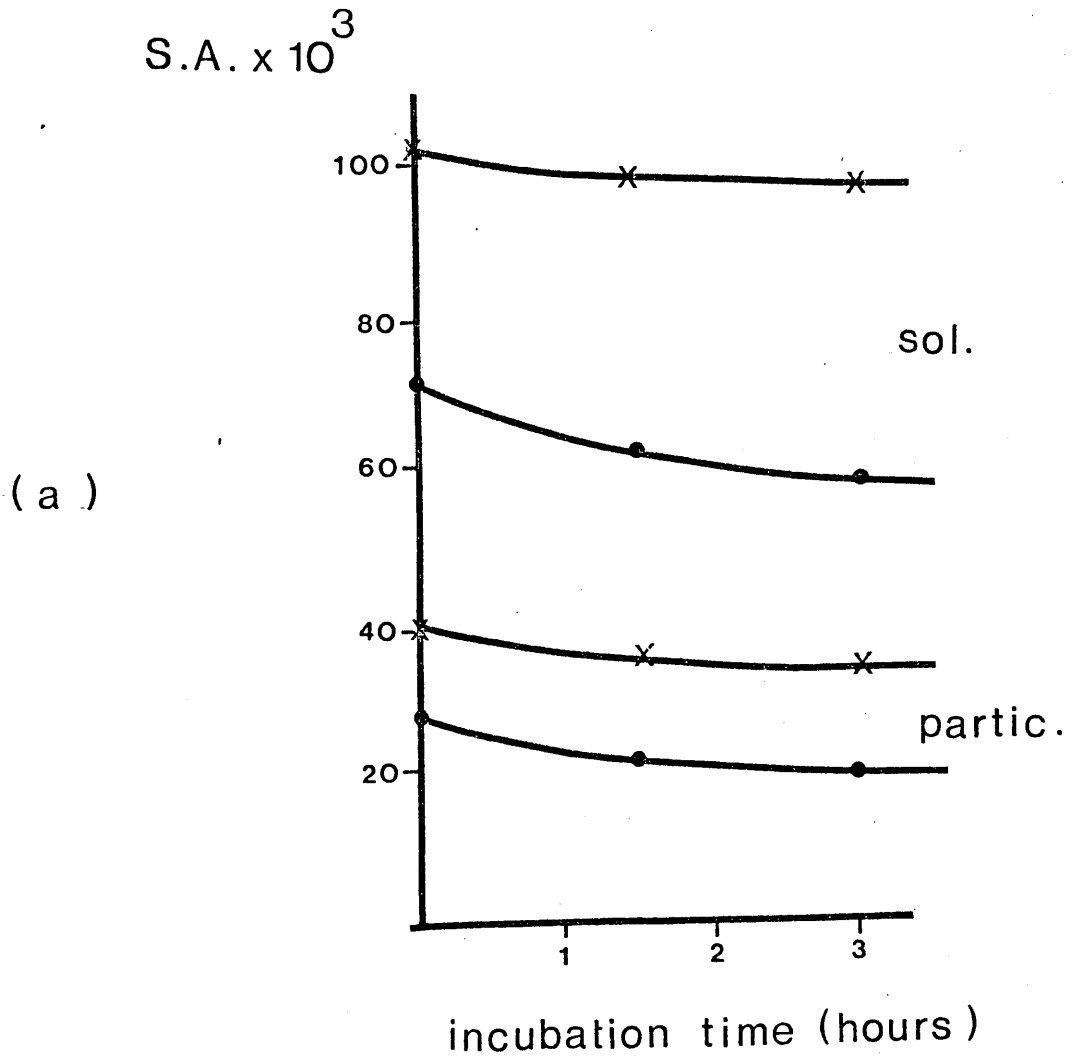


Figure 5.16

Decay of CB-activity at 37^o in the presence and absence of vincristine

Soluble and particulate extracts of rat brain were incubated at 37^o in the presence (X) and absence (●) of 0.5mM vincristine for various times (as shown). 2.5 μM [³H]-colchicine was then added and incubation continued for a further 1.5h. The samples were then assayed in triplicate by the filter disc method (see Chapter 2) to determine the amount of protein-bound radioactivity. The results presented are the means from 2 separate experiments. Results in (b) are extrapolated to zero time of incubation to correct for losses in CB-activity occurring during the incubation procedure.



containing 10mM phosphate buffer and 10mM $MgCl_2$ and was subjected to sonication for varying times (up to 25 minutes) in the cold. The homogenate was then centrifuged and the soluble and particulate fractions obtained were assayed for CB-activity. The particulate fraction obtained after sonication was found to contain 70% of the total recovered protein compared to 80% in the control particulate sample, but it accounted for about 80% of the recovered CB-activity compared to 50% in the control. This rise was apparently not due to any direct stimulation of particulate CB-activity as a result of the sonication procedure, as determined in control experiments. It therefore appeared that a selective partial redistribution of CB-protein into the particulate fraction had taken place, and this was again indicated when the samples were subjected to SDS-polyacrylamide gel electrophoresis, when the tubulin band appeared denser than in the control sample. This was the opposite effect to that sought.

Sonication of a frozen homogenate, in which the cell organelles would be lysed, gave the same pattern of results as those obtained when a fresh homogenate was used.

Sonication of a homogenate prepared in buffer containing Triton X-100 again gave similar results. It therefore appears a real possibility that CB-sites normally occluded in cell membranes had been unmasked by the sonication procedure, and were then free to bind colchicine.

Ionic detergents (e.g. sodium dodecyl sulphate, SDS) are also sometimes used in the extraction of membrane-bound proteins. The method of Grossfeld and Shooter (1971) which incorporates lysis, Triton extraction and SDS extraction of the particulate fraction was used, in a modified form, to minimise loss of CB-activity in the preparation. A homogenate was prepared in 0.32M sucrose containing 10mM phosphate buffer and 10mM $MgCl_2$, and this was centrifuged for 10^5 g-h to obtain the particulate fraction. This was subjected to osmotic shock by suspending it in water to lyse the intact cell organelles present. The suspension was then centrifuged for a further 10^5 g-h. The pelleted material obtained was resuspended in homogenising buffer containing 1% Triton and this was recentrifuged. Finally, the pelleted material obtained after this extraction was suspended in homogenising buffer containing 1% SDS at room temperature (as the SDS was found to come out of solution in the cold), and after another centrifugation only a very small amount of pelleted material remained. This contained a small amount of protein which had not been solubilised by the procedure used.

The soluble sample obtained which contained SDS was dialysed for 1-2 hours at room temperature, against 10mM phosphate-Mg²⁺ buffer, to remove the SDS as this had been found to substantially reduce CB-activity. The fractions obtained in this extraction were then assayed for CB-activity, and the results obtained are shown in Table 5.3. It was found that the initial high-speed supernatant fraction (in buffered sucrose) contained about half the total CB-activity recovered from the homogenate, and by far the highest CB-activity/mg protein. The SDS extract had about one third of the total recovered CB-activity, but almost half the total recovered protein as well, indicating that it also contained many other non-CB-proteins. However, this procedure was found to be the most satisfactory in solubilising protein which retained its CB-activity. With further work it should be possible to purify the membrane-bound CB-protein by conventional techniques, using this preparation as a starting material.

It was apparent from the results of the solubilisation experiments that the membrane-bound CB-activity was not easily released. Only about 15% of the particulate protein could be released by treatment with water and Triton; however, the bulk of the protein and CB-activity could be released using SDS. Clearly, a substantial amount of future investigation will be required to characterise this membrane-bound CB-protein.

Table 5.3

Solubilisation of membrane-bound CB-activity

Soluble and particulate extracts of rat brain were prepared and the particulate fraction was resuspended in water. This was recentrifuged, and the pelleted material was suspended in homogenising buffer containing 1% Triton X-100. The suspension was again centrifuged, and the pelleted material was finally suspended in homogenising buffer containing 1% SDS. This was again recentrifuged; the SDS-soluble fraction was dialysed, then all the extracts were assayed for CB-activity. The presence of SDS was found to partially inhibit colchicine-binding; this effect has been corrected for in the results shown.

Table 5.3

Solubilisation of membrane-bound CB-activity

<u>Fraction</u>	<u>Total protein(mg)</u>	<u>% recovered protein</u>	<u>CB-activity³ (bound cpm x 10³)</u>	<u>% recovered CB-activity</u>	<u>S.A.³ (x 10³)</u>	<u>R.S.A.</u>
Initial high-speed sucrose supernatant	17.8	20.6	1191	50.6	67	2.46
Subfractions of initial high-speed particulate fraction:						
Water-soluble	13.0	15.1	84	3.6	7	0.24
Triton-soluble	13.8	16.0	250	10.6	18	0.66
SOS-soluble	38.8	45.1	771	32.8	20	0.73
Final pellet	2.8	3.2	55	2.3	20	0.73
	<u>86.2</u>	<u>100.0</u>	<u>2351</u>	<u>99.9</u>		

CHAPTER SIX

Preparation and some properties
of purified brain CB-protein

Introduction

Colchicine-binding protein was purified from mammalian brain following a slightly modified procedure of the method used by Weisenberg et al (1968). Colchicine-binding activity of each of the subfractions obtained in the procedure was determined, in order to check the purification of the protein in the preparation. Each of the subfractions was also subjected to polyacrylamide gel electrophoresis, so that the protein content (on a molecular weight basis) could be monitored throughout the procedure. An amino acid analysis was also performed on the purified protein obtained, and estimation of the sugar content of the protein was attempted.

It is a possibility that CB-protein ('microtubule protein') is concerned with neurotransmitter release, since if cells are treated with colchicine, this drug binds to the microtubules, and neurotransmitter release at the synapse is inhibited. The transmitter is stored in small vesicles in the nerve-endings, and these are thought to move to the surface membrane of the cell when stimulated. This is accompanied by a rise in adenosine 3':5' -cyclic monophosphate (cAMP) concentration. One recent theory of the action of cAMP concerns its ability to stimulate specific protein kinases (Kuo and Greengard, 1969). A cAMP-dependent protein kinase has been purified from bovine brain (Miyamoto et al (1969). The usual assay for this enzyme is based on its ability to stimulate the phosphorylation of either histone or casein, but other substrates may also be used. Goodman et al (1970) recently found that purified brain CB-protein could serve as a substrate for an 'intrinsic' cAMP-dependent protein kinase which was closely associated with the protein. The purified brain CB-protein prepared was therefore used to confirm and extend the results already published.

Materials and Methods

Pig brain was obtained within about 1h. after death from Walls', Wembley, and was transported, packed in ice, to the laboratory. Generally 600g - 1000g of brain was used. All operations during the purification procedure were performed at 4°C. The superficial blood vessels and meninges were first removed, then the tissue was minced with scissors and was washed twice by suspending in twice its volume of sucrose buffer (0.24M sucrose containing 10mM phosphate buffer, pH 6.8, and 10mM MgCl₂). The mince was strained through three layers of cheesecloth and was suspended in its own volume of sucrose buffer containing 0.1mM GTP. This was homogenised in a Waring blender for 30 seconds at top speed and was then centrifuged

at 23,000g for 20 min. The soluble fraction obtained was brought to 32% saturation with ammonium sulphate (adding 177g per litre of supernatant), by gradually adding the salt, with constant stirring, over about 15 min. The suspension was left to stand for 10-15 min. then was centrifuged at 23,000g for 20 min. This precipitated proteins poor in CB-activity. The supernatant fraction produced was then brought to 43% saturation with ammonium sulphate (adding a further 71g of ammonium sulphate per litre), then was left to stand and centrifuged as previously. The precipitate obtained was considerably enriched in CB-activity and was redissolved in about 50ml phosphate-Mg-GTP buffer per litre of original homogenate. This was added to about 200ml of packed DEAE-Sephadex (A-50) (made by swelling 5g, which was then washed with acid, then alkali, and left to equilibrate in phosphate-Mg buffer). The mixture was stirred intermittently for about 30 min. to allow the protein to adsorb, then the Sephadex was separated on a large Buchner funnel. The Sephadex was then washed with five times its volume (i.e. 1 litre) of phosphate-Mg-GTP buffer containing 0.4M KCl, stirring intermittently for 10 min. to elute some of the adsorbed protein. The mixture was filtered on the Buchner, then the Sephadex was washed twice with 100 ml phosphate-Mg-GTP buffer containing 0.8M KCl, again allowing about 10 min. per wash. This eluant was brought to 43% saturation with enzymic grade ammonium sulphate and the precipitated proteins were collected by centrifuging at 35,000g for 20 min. This was dissolved in about 20ml phosphate-Mg-GTP buffer and was dialysed overnight to remove excess ammonium sulphate and KCl. Long exposure to KCl was found to inhibit CB-activity, so the final precipitation served both to concentrate the protein and remove it from the KCl environment. The dialysed protein was then lyophilised, and was stored at -20°C .

The protein content of each of the fractions was determined by the method of Lowry et al (see Chapter 2), making corrections for the presence of ammonium sulphate where it affected the determination.

Samples of each fraction were incubated with $2.5 \mu\text{M}$ $[\text{}^3\text{H}]$ -colchicine (protein concentration up to 1mg/ml) for 1.5h. at 37°C . CB-activity was then determined by the filter-disc assay (see Chapter 2). The dialysed purified preparation was used fresh; the other fractions had been frozen overnight.

Polyacrylamide gel electrophoresis was performed on the fractions using 10% gels and phosphate-SDS buffer (see Chapter 2), loading between 50 μg and 200 μg protein/gel. The gels were fixed and stained with Coomassie brilliant blue.

For the amino acid analysis duplicate samples of 0.4mg lyophilised purified protein were hydrolysed in 0.5ml 6N HCl in sealed, evacuated tubes at 110°C for 25h. and 44h. The amino acid composition was determined using a Beckman amino acid analyser. L-norleucine was added to the hydrolysate to act as an internal standard. To correct for breakdown of the serine and tyrosine residues, the 25h. and 44h. hydrolysis results were extrapolated to zero time. Tryptophan, which is destroyed by this treatment, was determined spectrophotometrically by the method of Goodwin and Morton (1946).

Total sugars were estimated in purified CB-protein and in standard glycoprotein samples by a colorimetric method using indole as the colour developer (see Methods in Enzymology III p73). To 1 ml of an aqueous sample containing 5-25 µg sugar was added 2ml concentrated (98%) H₂SO₄, on ice, with shaking. 0.1ml of a 1% solution of indole in absolute alcohol was added, and the mixture placed in a boiling water bath for 10 min. The sample was cooled, then the colour intensity read at 480 nm. The samples were yellow in colour, and the blanks colourless. The colour was stable and could be read at any time.

Purified CB-protein and standard glycoprotein samples were also subjected to polyacrylamide gel electrophoresis using 7.5% gels and phosphate-SDS buffer (see Chapter 2). Duplicate sets of gels were prepared, one of each set being fixed and stained with Coomassie brilliant blue to show the positions of the protein bands. The remaining gels were stained for carbohydrate by the modified periodic acid-Schiff (PAS) technique of Zacharius et al (1969), as follows:

- (i) gels were immersed in 5% TCA for 30 min. then were rinsed with distilled water
- (ii) immerse in 1% periodic acid in 3% acetic acid for a maximum of 50 min.
- (iii) wash six times for 10 min. each in 200ml water/gel, with shaking, or overnight with a few changes. The fourth wash is checked for the absence of iodate with 0.1M AgNO₃. Two further washes are then given.
- (iv) immerse in fuchsin-sulphite stain, in the dark, for 50 min.
(see below for preparation of the stain)
- (v) wash three times with freshly prepared 0.5% metabisulphite solution, 10 min. each
- (vi) wash with distilled water to remove excess stain, with shaking, overnight. The background clears leaving pink carbohydrate bands. The gels are stored in 7% acetic acid.

The fuchsin stain was prepared following the method of McGuckin and McKenzie (1958). 4g potassium metabisulphite was dissolved in 5ml concentrated HCl and made up to 500ml with water. When dissolved, 2g basic fuchsin was added (Gurr Ltd, London, SW6), stirring gently with a mechanical stirrer for 2 h. The solution was then left to stand for a further 2 h. A small amount of decolourising charcoal was then added (Norit A - Sigma) and the solution was filtered within 15 min. The resulting reagent was colourless and was stored for several months at 4°C.

Protein kinase activity was assayed by an adaptation of the method of Miyamoto et al (1969) and the filter disc method of Tao et al (1970). Protein samples (up to 100 µg) were preincubated in a final volume of 0.2ml in 0.1M tris-HCl buffer, pH 7.5, containing 20mM MgCl₂, for 5 min, at 30°C. γ -³²P-labelled ATP (10 µl), containing about 10⁶ cpm, was then added to give a final concentration of 250 µM (as method of Tao) or 5 µM (as method of Miyamoto) and the samples were reincubated for a further 10 min. If cAMP was to be included, a final concentration of 25 µM was used and, where extrinsic kinase activity was to be measured, 40 µg histone was added to the incubation mixture prior to the addition of the ATP. The reaction was terminated by the addition of 1ml ice-cold 15% TCA, and 0.3ml of 0.1% BSA was added to co-precipitate the protein. The mixture was left to stand on ice for a few minutes then was filtered through Millipore glass fibre discs (GF/A, 2.5cm diameter). The filter was washed rapidly (in about 30 sec.) with 40ml ice-cold 5% TCA. This process did not remove any contaminating lipids (which have since been shown to be labelled under similar conditions; however, they make up only a minor proportion of the total bound label). The disc was placed in 5ml of Bray's scintillant (see Chapter 2) and was counted directly. The efficiency of counting ³²P was approximately 100%, as found by an internal standardisation method.

100 µg samples of ³²P-labelled purified CB-protein were also precipitated with TCA, then were processed for electrophoresis using the tris-SDS-urea buffer system (see Chapter 2). Samples incubated with labelled ATP in the presence and absence of added cAMP were used and duplicate gels were processed. The gels were then sliced and dried (see Chapter 2) and the slices were left in the dark, in contact with an X-ray film, for 3 days. This produced an autoradiograph of the separation of labelled proteins from the sample.

A phosphokinase enzyme preparation was carried out, as described by Miyamoto et al, for use in the kinase assays. The starting material was rat, adult rabbit or foetal rabbit brain, which was homogenised in a Waring blender and then centrifuged at 27,000g for 30 min. at 4°C. All subsequent steps were performed in the cold. The supernatant obtained was adjusted to pH 4.8 by the dropwise addition of 1M acetic acid, with stirring. After standing 10 min. the precipitate was removed by centrifuging at 27,000g for 30 min. The pH of the supernatant was then readjusted to 6.5 with 1mM potassium phosphate buffer, pH 7.0, containing 2mM EDTA. The resulting solution was then dialysed against 20 volumes of the same buffer overnight. The dialysed preparation was then centrifuged at 27,000g for 30 min. producing a soluble, partly purified preparation. A reported 10-fold purification of the enzyme is obtained (Miyamoto et al) at this stage. 3g of brain routinely produced approximately 5mg of this partly purified protein.

Binding of cAMP to purified CB-protein was performed using a modified version of the method of Tao (1971). Up to 100 µg protein was used in 10mM tris-HCl buffer, pH 7.5, containing 4mM MgCl₂. 77 pmoles of cyclic (³H)-AMP was added (containing approximately 0.4 x 10⁶cpm) and the mixture, in a total volume of 0.2ml, was left on ice for 20 min. Binding was stopped by dilution with 0.8ml buffer and the protein was filtered off on an ice-cold, presoaked Millipore cellulose filter (pore size 0.45 µ), washing the filter with 15ml buffer. The disc was then dissolved in 5ml Bray's scintillant (see Chapter 2) and was counted directly. The efficiency of counting was found to be 48% by an internal standardisation method.

Radioactive [γ -(³²P)]-ATP and cyclic-(³H)-AMP were obtained from the Radiochemical Centre, Amersham, Bucks.

Results

(a) Purification of brain CB-protein

Results obtained concerning distribution of CB-activity and protein recovery in the purification procedure are given in Table 6.1. As expected, introduction of KCl into the samples inhibited CB-activity, but this was altered after removing the protein from the KCl environment (final fraction). It is also noteworthy that some three-quarters of the total brain CB-activity is present in the first pellet. This finding generally agrees with results obtained using other mammalian brain tissue (see Chapter 3), and also led to work involving release of membrane-bound CB-protein (see Chapter 5).

Table 6.1

Recoveries of protein and CB-activity during the purification of brain CB-protein

Results were obtained using 1100g pig brain, purified as described in Methods. Protein and CB-activity determinations were performed as described in Chapter 2.

Table 6.1

Recoveries of protein and CB-activity during the purification of brain CB-protein

<u>Fraction</u>	<u>Total CB-activity</u> ($\times 10^6$ cpm)	<u>% recovered CB-activity</u>	<u>Total protein(mg)</u>	<u>% recovered protein</u>	<u>S.A.</u> ($\times 10^3$)	<u>Purification</u> (homog. = 1)
1. 1st supernatant	360	23	10,920	12	33	2
2. 1st pellet	$\frac{1186}{1546}$	77	$\frac{83,160}{94,080}$	88	14	1
3. 32% ammonium sulphate supernatant	353	99	7,840	76	45	3
4. 32% ammonium sulphate pellet	$\frac{4}{357}$	1	$\frac{2,450}{10,290}$	24	14	1
5. 43% ammonium sulphate supernatant	91	26	5,600	78	16	1
6. 43% ammonium sulphate pellet	$\frac{252}{343}$	74	$\frac{1,606}{7,206}$	22	152	11
7. 0.4M KCl supernatant	3	81	1,540	92	2	0.1
8. 0.8M KCl supernatant	$\frac{0.7}{3.7}$	19	$\frac{135}{1,675}$	8	5	(see text) 0.3
9. 43% ammonium sulphate supernatant	0.7	2	28	36	3	0.2
10. 43% ammonium sulphate pellet	$\frac{30}{30.7}$	98	$\frac{49}{77}$	64	630	45

The purification procedure was often scaled down to handle conveniently up to 50g of brain (usually rabbit). Greater yields were obtained from foetal rabbit brain (on a weight basis) and several mg purified protein were usually obtained. From the results, a relatively pure protein preparation had been produced which had a much higher relative CB-activity than the starting material.

Results of electrophoresis of the fractions obtained during the purification procedure are shown in Fig. 6.1. It can be seen that all the fractions contained a tubulin-comigrating band, and that the majority of the protein recovered in the 0.8M KCl fraction corresponded to this 60,000 molecular weight.

(b) Amino acid analysis of purified CB-protein

The amino acid composition of the purified pig brain CB-protein is given in Table 6.2, together with data published for CB-proteins from different sources. It is evident that the compositions are similar. The main differences are that glycine and serine in the purified pig brain protein are present in a rather higher proportion than in CB-protein from other sources, whereas leucine and proline are present in a lower proportion. A method for comparing similarity of proteins (Marchalonis and Weltman (1971)) was used to compare the relatedness of these proteins, involving summing the squares of the differences for each pair of amino acids considered in the proteins. The result is a number; obviously, the greater the differences in composition, the greater this number will be. If the proteins are unrelated, the number obtained is 100 or more; if the proteins are related in some way (i.e. parts of the sequence are similar), the number obtained in the comparisons is 50 or less. The results obtained comparing the compositions of the different CB-proteins are summarised in Table 6.3. It would appear from this type of comparison that all the proteins are similar.

A calculation on the helical content of the pig brain purified CB-protein (method of Havsteen, 1966) which is based on the proportion of serine + threonine + proline (helix breakers) in the protein gave a value of 18.5%, which is calculated to result in a helical content in the protein of 22.7%. The percentage of helix breakers in the other proteins is found to be similar.

(c) Sugar content of purified CB-protein

The sugar content of purified pig brain CB-protein was determined quantitatively, and the results are given in Table 6.4. The value of 3.3% carbohydrate in the protein compares reasonably with the value of 1.3% published later by Margolis and Shelanski (1972).

Figure 6.1

Electrophoresis of samples obtained in the purification procedure of tubulin from rabbit brain

Brain tubulin was purified according to the procedure described in Methods. Samples of each fraction (containing 100-200 μg of protein) were applied to the gels, and electrophoresis was carried out in phosphate-SDS buffer (see Chapter 2). The gels were then fixed, and stained with Coomassie brilliant blue.

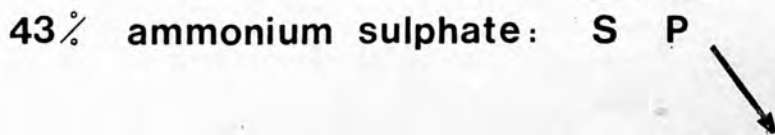
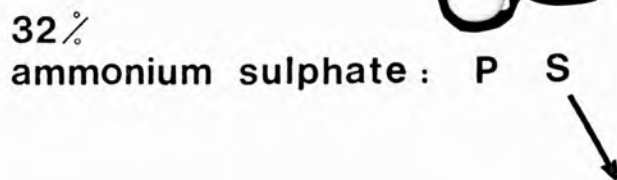
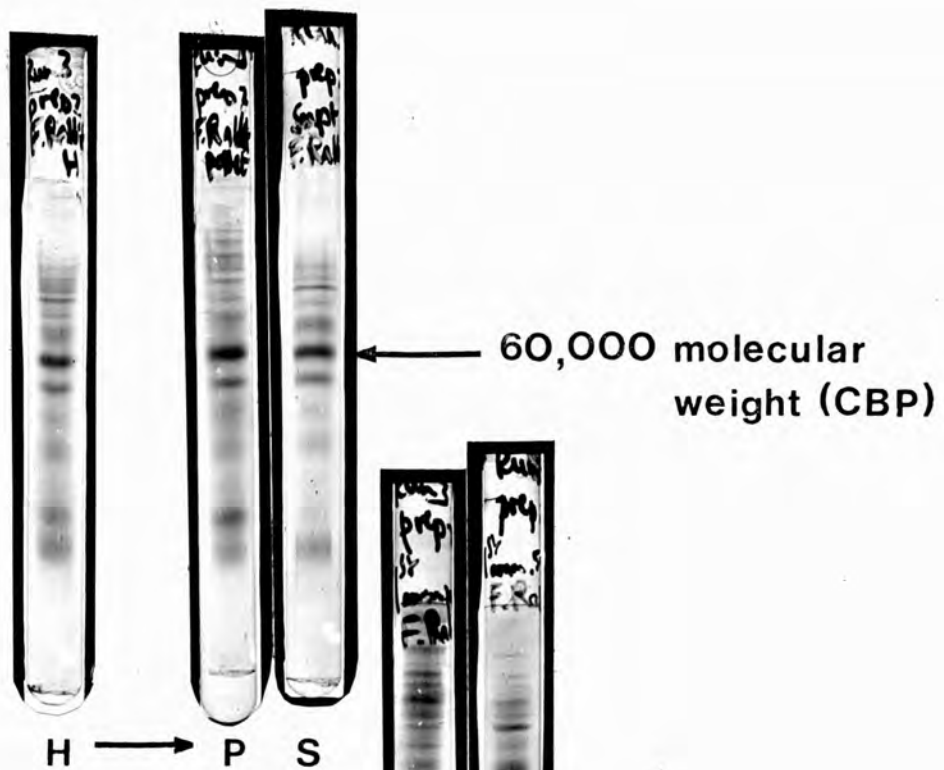


Table 6.2

Amino acid compositions of CB-proteins from various sources

Purified pig brain CB-protein was prepared and its amino acid composition determined as described in Methods. Origins of the remaining CB-proteins are as follows:

- (a) Pig brain purified CB-protein (Weisenberg et al, 1968).
- (b) Alkylated purified calf brain CB-protein (Falxa and Gill, 1969).
- (c) Sea urchin sperm tail CB-protein (Shelanski and Taylor, 1968).
- (d) Squid giant axon CB-protein (Huneeus and Davison, 1970).
- (e) Neuroblastoma CB-protein (Olmsted, 1970).

Values given are expressed as moles %.

Table 6.2

Amino acid compositions of CB-proteins from various sources

Amino acid	Pig brain CB-protein		(a)	(b)	(c)	(d)	(e)
	Residues/ 60,000 Mwt.	Mole %					
Ala	38	8.1	7.5	9.2	7.7	7.4	8.1
Arg	21	4.5	4.4	4.1	5.1	5.0	5.6
Asp	45	9.5	10.3	11.5	9.6	10.6	10.1
Cys- $\frac{1}{2}$	5	1.1	1.9	1.1	1.5	2.4	1.8
Glu	70	14.8	14.2	13.1	14.1	13.0	13.4
Gly	45	9.5	7.9	6.3	7.9	8.4	8.2
His	12	2.6	2.4	3.4	2.8	2.8	2.4
Ileu	23	4.9	4.5	5.2	4.5	4.5	5.0
Leu	28	5.9	7.7	8.7	7.9	7.4	8.7
Lys	17	3.6	3.9	6.2	6.1	4.6	6.5
Met	15	3.1	2.3	0.9	2.5	2.9	2.3
Phe	21	4.4	4.8	3.6	3.5	4.1	4.0
Pro	13	2.8	5.2	5.4	5.2	5.3	5.2
Ser	46	9.8	5.9	5.6	5.3	6.8	5.5
Thr	28	6.0	6.3	6.1	6.2	5.4	6.4
Try	3	0.7	0.7	not detected	0.8	0	not detected
Tyr	8	1.6	3.9	2.7	3.0	3.4	3.0
Val	34	7.1	6.3	7.0	6.3	5.8	6.6
	<u>472</u>						

Table 6.3

Comparison of the relatedness of CB-proteins from various sources

The amino acid compositions of the various CB-proteins shown in Table 6.2 were used, and pairs of the proteins were compared using the method of Marchalonis and Weltman (see text). The difference between each pair of amino acid values was squared and the squares were summed. The sums obtained are given in the Table. Proteins with similar compositions will thus give low values for the sum of the (differences)².

Table 6.3

Comparison of relatedness of CB-proteins from various sources

	<u>Purified pig brain CB-protein</u>	<u>Pig brain CB-protein (Weisenberg)</u>	<u>Alkylated calf brain CB-protein</u>	<u>Sperm tail CB-protein</u>	<u>Squid axon CB-protein</u>
Pig brain CB-protein (Weisenberg)	36	-	-	-	-
Alkylated calf brain CB-protein	65	22	-	-	-
Sperm tail CB-protein	44	9	15	-	-
Squid axon CB-protein	33	7	24	10	-
Neuroblastoma CB-protein	50	12	13	3	11

Table 6.4

Quantitative determination of sugar content of proteins

Up to 25 μg sugar was used in this determination, which is a colorimetric method employing indole as the colour developer (see Methods). Within this range, a linear standard curve was obtained. Pig brain CB-protein was purified as described in Methods.

Table 6.4

Quantitative determination of sugar content of proteins

<u>Sample</u>	<u>µg carbohydrate</u>	<u>µg protein</u>	<u>% carbohydrate</u>
casein (a standard)	13	292	4.5 (theoretical = 5%)
purified CB-protein	5	150	3.3

Electrophoresis of casein and ovomucoid (both glycoproteins) and purified pig brain CB-protein produced a protein band which stained blue in one set of gels, and a carbohydrate band which stained pink in a second set of gels. The protein and carbohydrate bands for each substance occupied approximately the same positions on the gels, indicating that the carbohydrate moieties are part of the protein molecules. The carbohydrate stain gave a negative result in the absence of sugars, and this was demonstrated using a control, lactoglobulin, which is a non-glycoprotein.

(d) Enzyme experiments involving purified CB-protein

Results of the determinations of intrinsic and extrinsic kinase activity of the partly purified enzyme preparations are given in Fig. 6.2, in which the time of incubation with labelled ATP was varied. Extrinsic activity results for an incubation time of 10 min. were linear when the enzyme concentration was varied in the range 0 - 100 μg . The optimal pH for the reaction was around 7.5; a drop of approximately 20% in the enzyme activity was found at pH 6.4 and at pH 8.2.

In a limited number of experiments it was also found that the purified pig brain CB-protein showed intrinsic kinase activity (see also Fig. 6.2). It would therefore seem that the CB-protein contained a closely associated kinase, which could phosphorylate the protein. The enzyme activity was next determined in the presence of added cAMP to find if a stimulation of activity was produced. A small stimulation was found, confirming the results of Goodman et al (1970). The maximum amount of ^{32}P which could bind to the CB-protein was found to be approximately 50 pmoles/100 μg , i.e. 30 nmoles/60,000g (= 0.03 mole P/mole protein).

The partially purified kinase preparations were next used to find if they could phosphorylate the purified CB-protein. The results are shown in Fig. 6.3. These indicate that the CB-protein can act as a substrate for the phosphorylation, although it was not phosphorylated as much as was the histone. The incorporation of ^{32}P into the CB-protein was increased in the presence of added cAMP.

It has been reported that there is a requirement for divalent metal ions for the phosphorylation process to occur (Miyamoto et al), the stimulation of kinase activity being greatest in the presence of Mg^{2+} , Mn^{2+} or Co^{2+} ; Ca^{2+} , however, strongly inhibiting the enzyme activity. Since the CB-protein was prepared in buffers containing Mg^{2+} ions, the lowest concentration which could be used in the enzyme experiments was 1mM.

Figure 6.2

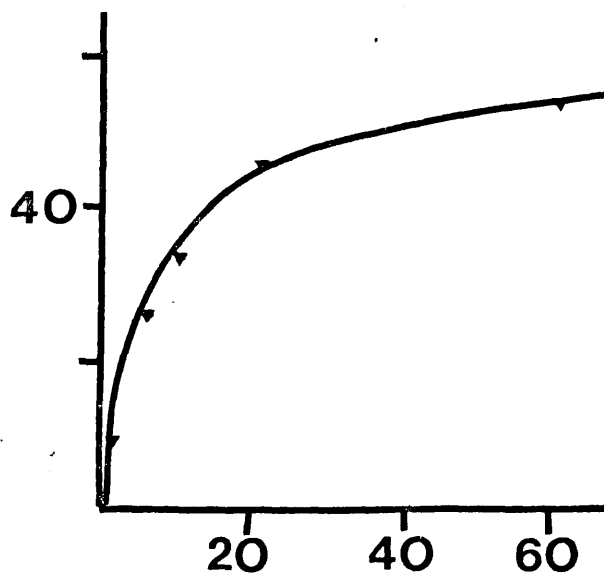
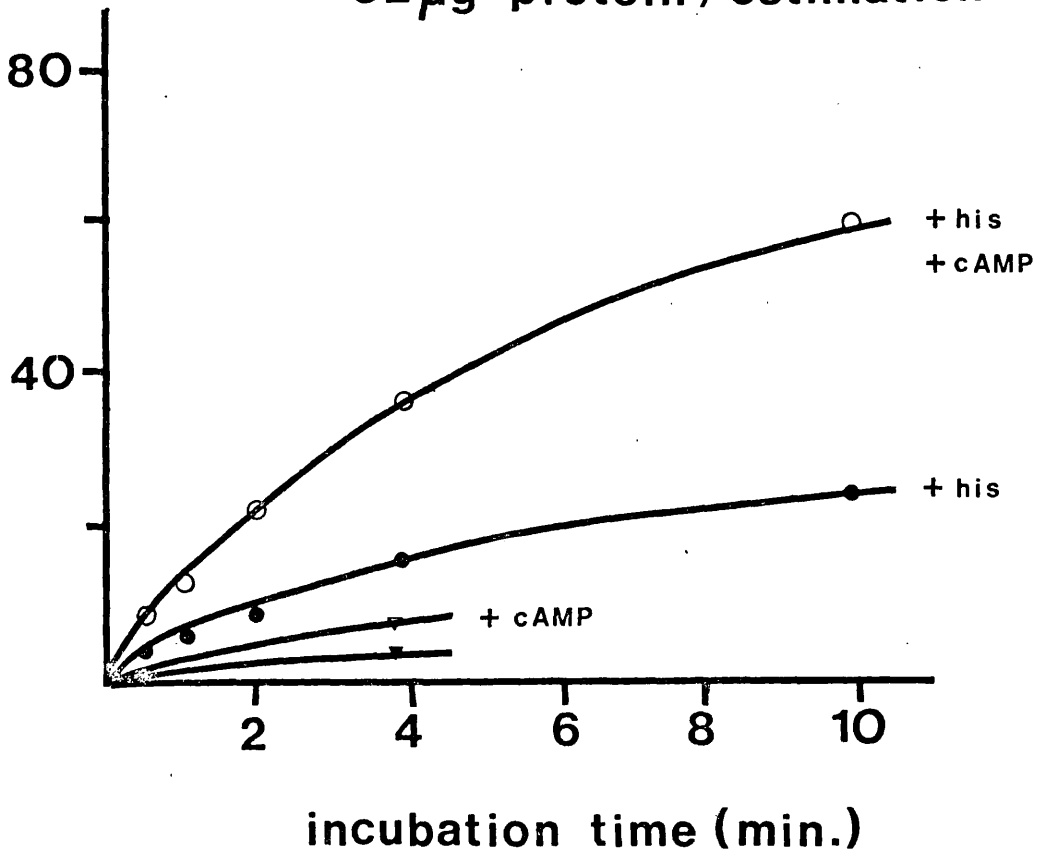
Protein kinase activity of (a) partially purified enzyme from rat brain and (b) intrinsic kinase activity of pig brain purified CB-protein

Protein samples were incubated in 0.2 ml of 0.1M tris-HCl buffer, pH 7.5, containing 20mM MgCl₂ and 5 μM [γ-³²P]-ATP for the times indicated at 30°. Where used, cAMP was 25 μM final concentration; the amount of histone used was 40 μg/tube. The protein was then precipitated by TCA, and was removed from the mixture by Millipore filtration using glass fibre discs. The protein was washed on the filter with cold TCA and was transferred with the disc to 5ml of Bray's scintillant, and was counted. The results are corrected for blanks obtained using heat-denatured protein samples.

pmoles
bound
 ^{32}P

(a)

52 μg protein/estimation



(b)

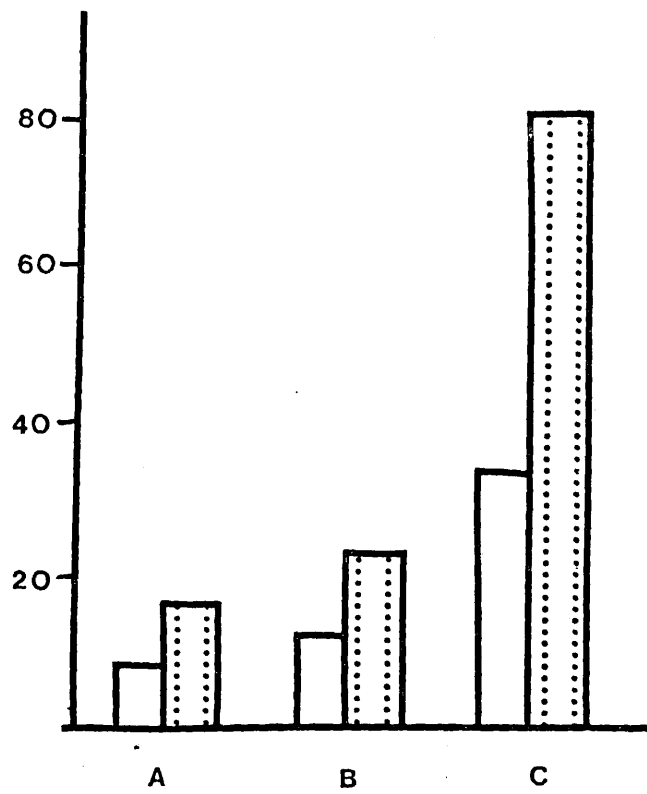
100 μg protein/
estimation

Figure 6.3

Phosphorylation of histone and purified CB-protein
by a partly purified kinase preparation

Protein samples were incubated in 0.2ml of tris-HCl buffer, pH 7.5, containing 20mM MgCl₂ and 5 μM [γ -³²P]-ATP for 10 minutes at 30°, in the absence () and presence (.....) of 25 μM cAMP. The protein was precipitated by addition of TCA, and was removed from the mixture by Millipore filtration using glass fibre discs. The precipitate was washed with cold TCA and was transferred with the disc to 5ml of Bray's scintillant, and was counted. The results are corrected for blanks obtained using heat-denatured protein samples.

PMOLES
BOUND ³²P



A = rat kinase (52 μ g)

B = rat kinase + purified pig CB-protein (100 μ g)

C = rat kinase + histone (40 μ g)

However, this concentration did not give a significantly different result compared to that obtained using a concentration of 10mM Mg^{2+} . Also, addition of 10mM Ca^{2+} or 5mM Mn^{2+} had no effect on the enzyme activity. This property therefore appears rather different to that of the soluble kinase of Miyamoto et al.

The addition of colchicine is known to inhibit neurotransmitter release, which is thought by some workers to involve phosphorylation reactions. The effect of added colchicine on the kinase activity of purified CB-protein was therefore investigated. Other alkaloids (vincristine and vinblastine) were also used, at varied concentrations. The results obtained are shown in Fig. 6.4, from which it is apparent that in the absence of cAMP, colchicine doubled the kinase activity, but the other alkaloids had very little effect. However, in the presence of cAMP, the highest concentrations of all the alkaloids stimulated the kinase activity. (It may be noteworthy that the alkaloids above a concentration of $10^{-5}M$ cause aggregation of CB-proteins (Weisenberg and Timasheff, 1970); this could possibly act as a better phosphorylating substrate than the unaggregated material, selectively increasing the incorporation of phosphate from ATP into CB-protein.)


The results of the electrophoresis and autoradiography of ^{32}P -labelled pig brain purified CB-protein (in the presence and absence of cAMP) are shown in Fig. 6.5. It can be seen that most of the ^{32}P incorporated was present in the major tubulin bands (molecular weight 58,000 and 55,000) and these were each labelled to approximately the same extent. This both confirms and extends the results of Goodman, since in this buffer system (tris-SDS-urea) the CB-protein is resolved into two molecular weight species, and these are both shown to be phosphorylated.

(e) cAMP binding to purified CB-protein

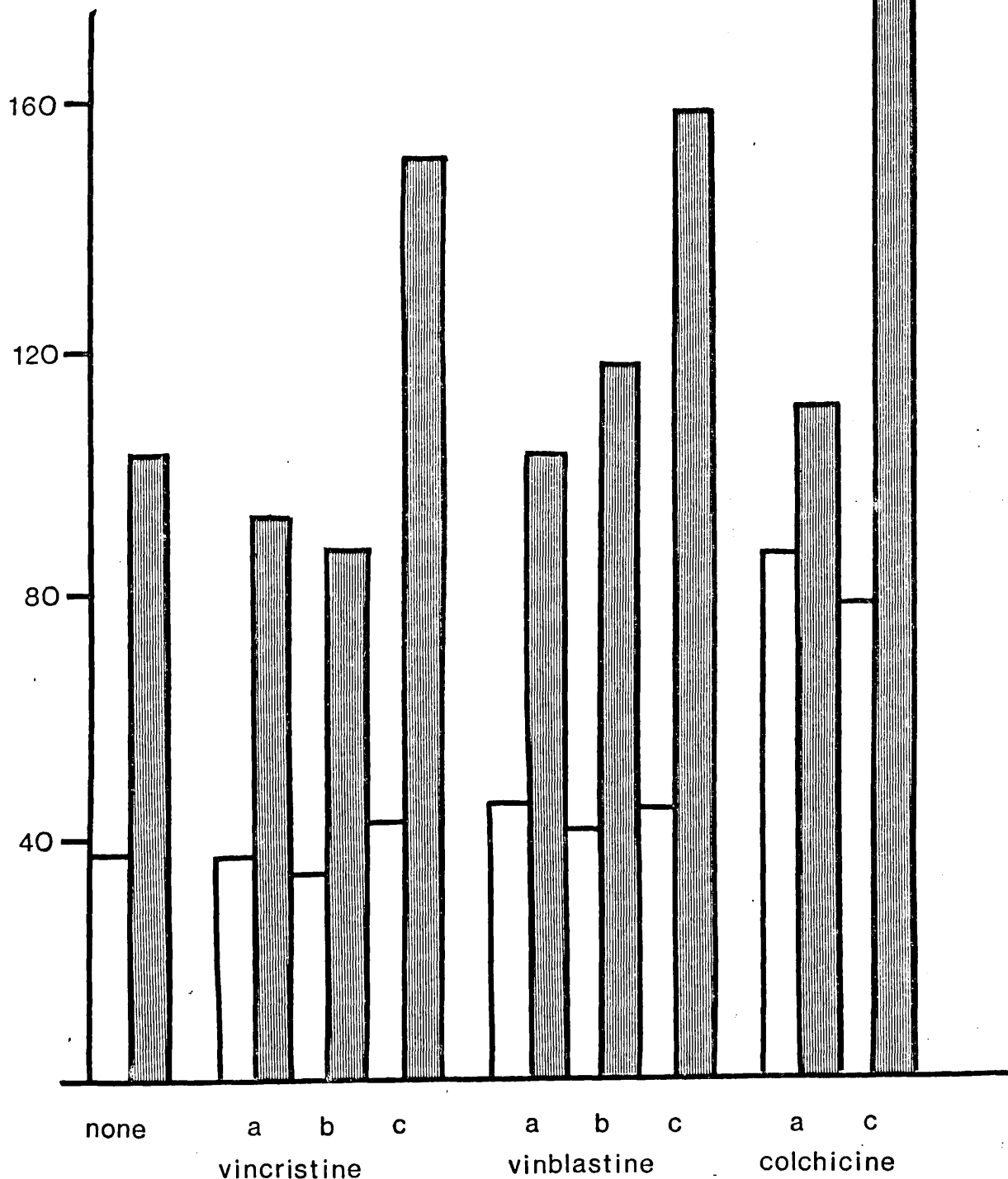
The results obtained from cAMP-binding assays using purified pig and foetal rabbit brain CB-protein are shown in Fig. 6.6. Varied concentrations of colchicine and the Vinca alkaloids were also added to test their effects. It was calculated that between 0.2 and 1.5 mmoles of cAMP were bound per mole (60,000g) of CB-protein. It was also found that all the alkaloids, at the concentrations used, slightly inhibited cAMP-binding. These observations again confirm and extend the results obtained by Goodman et al (1970).

Figure 6.4

Intrinsic kinase activity of purified pig brain
CB-protein in the presence of various alkaloids

100 μg of purified pig brain CB-protein was incubated in 0.2ml of tris-HCl buffer, pH 7.5, containing 20mM MgCl_2 and 250 μM $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ for 10 minutes at 30° in the absence () and presence () of 25 μM cAMP. Other additions were as indicated in the figure. The protein was precipitated by TCA and was filtered off using Millipore glass fibre discs. The precipitate was washed with cold TCA and was transferred with the disc to 5ml of Bray's scintillant, and was counted. The results are corrected for blanks obtained using heat-denatured protein samples.

pmoles
bound ^{32}P



ADDITIONS

a = 10^{-6} M
b = 10^{-5} M
c = 10^{-4} M

Figure 6.5

Electrophoresis and radioautography of phosphorylated pig brain purified CB-protein

Pig brain purified CB-protein was phosphorylated in vitro using $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$. It was then electrophoresed using the tris-SDS-urea buffer system (see Chapter 2), and the gels were fixed and stained, then sliced and dried (see Chapter 2). The slices were placed in contact with X-ray film for 3 days to obtain the radioautograph.

(a) (b)
Photograph Radioautograph
of gel slices

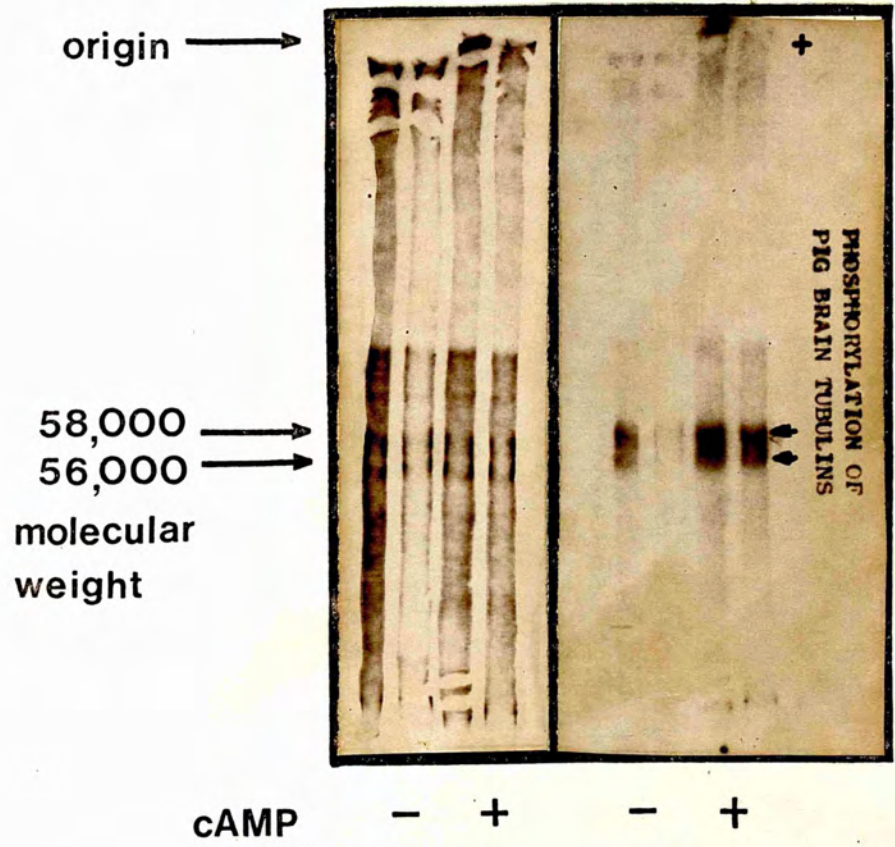


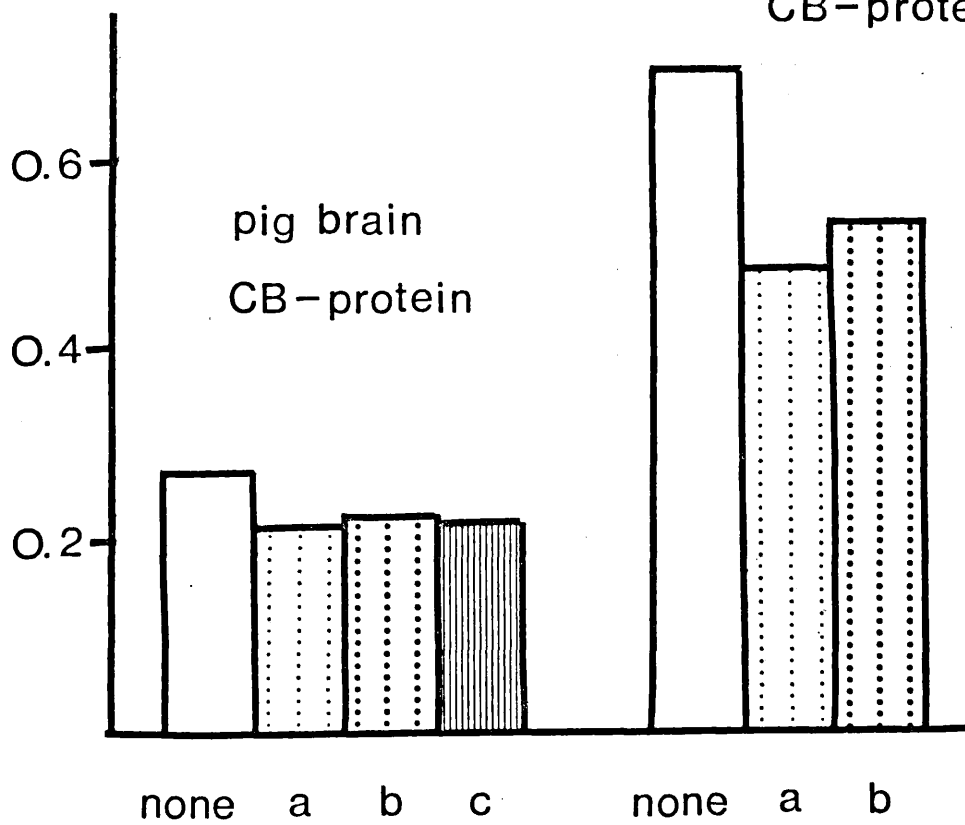
Figure 6.6

Effects of antimetabolic drugs on the cAMP-binding activity of purified brain CB-protein

100 μ g of pig brain purified CB-protein or 35 μ g of foetal rabbit purified CB-protein were incubated in 0.2ml of 10mM tris-HCl buffer, pH 7.5, containing 4mM $MgCl_2$ and 77pmoles of cyclic- $[^3H]$ -AMP, at 4 $^{\circ}$ for 20 minutes. Other additions were as indicated. The mixture was then diluted with 0.8ml of cold buffer and the protein was filtered off using a Millipore cellulose filter. This was washed with cold buffer and the protein and disc were placed in 5ml of Bray's scintillant and counted directly.

pmoles cAMP
bound

foetal rabbit
CB-protein



ADDITIONS

a = 10^{-6} M colchicine
b = 10^{-4} M vincristine
c = 10^{-4} M vinblastine

CHAPTER SEVEN

Isolation and some properties
of intact microtubules

Introduction

More direct evidence is needed to clarify the relationship between colchicine-binding protein and brain microtubules (neurotubules) (see Discussion). Brain is a rich source of CB-protein, which is considered to be the structural subunit of microtubules for various reasons, mainly due to the fact that colchicine, which disrupts microtubules, also binds to CB-protein (Borisy and Taylor, 1967; Wilson and Friedkin, 1967). It is therefore important to obtain additional evidence that CB-protein really is the subunit of brain microtubules. This problem was approached by attempting to purify intact microtubules and solubilise them, then studying some of their properties.

To isolate intact neurotubules their structures must be stabilised, since they depolymerise under the conditions used to separate subcellular particles (homogenisation of the tissue in sucrose buffer and subsequent fractional or density gradient centrifugation). A medium was therefore used which stabilised these structures. Kane, (1965) reported that a medium containing ethanol or hexylene glycol stabilised the microtubule structures of the mitotic apparatus of sea urchin eggs. The experiments of Kirkpatrick (1969), who used mammalian brain, confirmed this finding. An attempt was therefore made to isolate intact neurotubules from hexylene glycol homogenates, and to purify them by sucrose gradient centrifugation so that some of their properties, particularly CB-activity, could be studied. No work had been published concerning CB-activity of these preparations as the preserving medium apparently interfered with the binding reaction. This was therefore a very important property to investigate.

Another method, which substituted zinc ions in the buffer for the hexylene glycol, (Nickolson, Thesis, Leiden University) was also used to stabilise the neurotubular structures, permitting their isolation and further investigation of some of their properties.

Materials and Methods

Following the method of Kirkpatrick (1970), adult rat brain was homogenised in 5 parts of 20mM potassium phosphate buffer, pH 6.4, containing 1M hexylene glycol. The homogenate was centrifuged at 48,000g for 30 min. to pellet the larger organelles. A sucrose gradient was prepared containing 1ml of buffered hexylene glycol in 1.4M sucrose (density = 1.19g/ml) and 1ml of buffered hexylene glycol in 1.18M sucrose (density = 1.16g/ml).

2ml of the supernatant prepared was layered over this and the gradient was centrifuged at 140,000g for 1h. Material which had migrated to the 1.18-1.4M sucrose interface (which is reported to contain numerous microtubules) was collected in a syringe; the remainder of the samples in the tubes was pooled, and will be referred to as the gradient soluble fraction.

In Nickolson's preparation all the operations were carried out at 20° and a lower centrifugal force was employed. Immature brains were used (since these are said to contain more neurotubules than adult brain - see Peters and Vaughn, 1967) and a homogenate was made in 10mM imidazole-HCl buffer, pH 7.2, containing 1mM GTP and 1mM ZnCl₂. 4ml of the homogenate was layered over a gradient consisting of 6ml each of 2.0M, 1.8M, 1.5M, 1.3M and 0.8M sucrose in homogenising medium. A photograph of the gradients obtained after centrifugation at 13,000g for 30 min. is shown in Fig. 7.1. Samples from each of the interfaces were collected and portions were electrophoresed and assayed for CB-activity. The results of these determinations are expressed as specific activities (S.A.'s) which are cpm bound colchicine/mg protein; and as relative specific activities (R.S.A.'s) which are obtained from the relation

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

The latter facilitates comparison of CB-activities in the various sub-fractions with that of the parent fraction, whose R.S.A. is taken to be 1.

Electrophoresis of the samples was performed using 10% polyacrylamide gels and the phosphate-SDS buffer system (as described in Chapter 2).

Results

(a) Hexylene glycol preparations

The microtubule fraction obtained from the gradient, together with the gradient soluble fraction (see Methods) and a sample of the original supernatant were examined by electron microscopy for the presence of intact microtubules after fixing the samples with glutaraldehyde and negative staining with uranyl acetate on the grid. Numerous granules were seen in the presumed microtubular fraction, but no tubular structures could be detected in any of the samples.

Each of the samples was next dialysed for several hours at 4° against 10mM phosphate buffer containing 10mM MgCl₂ to remove the hexylene glycol.

Figure 7.1

Sucrose gradient separation obtained in the preparation of intact microtubules

Immature brain tissue was homogenised in 10mM imadazole-HCl buffer, pH 7.2, containing 1mM GTP and 1mM ZnCl₂. The homogenate was placed on the gradient and centrifuged at 13,000g for 30 minutes, when the separation shown was obtained. Results of the electrophoresis of the fractions obtained are given in Figure 7.3 and CB-activities are given in Table 7.2.



**sucrose
concentration**

0

0.8 M

1.3 M

1.5 M

1.8 M

2.0 M

They were then assayed for CB-activity; the results obtained are given in Table 7.1, from which it can be seen that the presumed microtubule-containing fraction was enriched in CB-activity more than five times in relation to the parent fraction (R.S.A. = 5.4).

Polyacrylamide gel electrophoresis of the samples gave the results shown in Fig. 7.2. It is apparent that the initial supernatant and the gradient soluble fractions contained many different proteins, but the microtubule fraction had one main protein band (of approximate molecular weight 60,000) and one other much fainter band (corresponding to an approximate molecular weight of 50,000). Purified CB-protein (see Chapter 6) is known to behave in an identical manner during this electrophoretic procedure.

Samples of each of the fractions were next treated with 1mM vinblastine and were incubated at 37° for 1h. This treatment is known to aggregate and precipitate CB-proteins (Wilson et al, 1970)(Weisenberg and Timasheff, 1970). The samples, now turbid, were then centrifuged and the precipitates and supernatants obtained were subjected to polyacrylamide gel electrophoresis. The vinblastine precipitates of the initial supernatant and the gradient soluble fraction were found to contain a main protein band (corresponding to a molecular weight of approximately 60,000) and two fainter bands (corresponding to a molecular weight of approximately 50,000 and 42,000). The vinblastine precipitate of the microtubule fraction gave the same separation pattern as the untreated microtubule fraction. Protein estimations on the samples showed that about half of the gradient soluble protein had been precipitated by vinblastine, while practically all the protein from the microtubule fraction had precipitated. When the vinblastine-precipitated protein was electrophoresed together with purified CB-protein on the same gel, the main bands coincided, indicating that the proteins in each sample had very similar molecular weights.

(b) Zn²⁺-GTP preparations

Fractions obtained from this separation were first examined by electron microscopy for the presence of intact microtubules after fixing the samples with glutaraldehyde, staining with osmic acid and embedding in epon. This procedure showed the presence of large numbers of tubules, apparently packed in parallel arrays.

Samples of each of the fractions obtained were also dialysed for several hours at 4° against 10mM phosphate buffer containing 10mM MgCl₂ to remove zinc ions, which had been found to interfere in the CB-assays. The samples were then each assayed for CB-activity; the results obtained are

Table 7.1

CB-activity of microtubule preparations made by
the hexylene glycol method

A soluble extract of rat brain homogenate was prepared in buffered hexylene glycol as described in Methods. This was subjected to sucrose gradient centrifugation using 1.18M sucrose and 1.4M sucrose in buffered hexylene glycol. The microtubule fraction was removed from the 1.18 - 1.4M sucrose interface; the remainder of the material was pooled as the gradient soluble fraction. Samples were dialysed against 10mM phosphate-Mg²⁺ buffer then were assayed for CB-activity. The results given were obtained using 1g of brain in the preparation. Results of electrophoresis of these fractions are shown in Figure 7.2.

Table 7.1

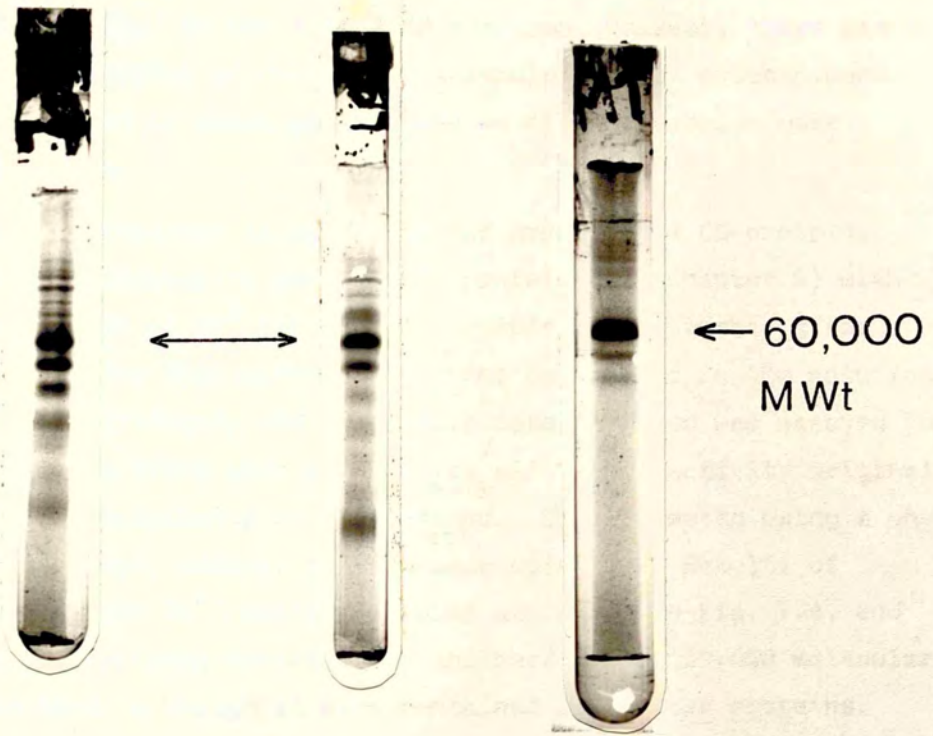
CB-activity of microtubule preparations made by the hexylene glycol method

<u>Fraction</u>	<u>Total protein(mg)</u>	<u>% recovered protein</u>	<u>CB-activity³ (bound cpm x 10³)</u>	<u>% recovered CB-activity</u>	<u>R.S.A.</u>	<u>S.A.³ (x 10³)</u>
Supernatant	37.2	-	390	-	-	10.5
Gradient soluble	32.1	98	234	89	0.9	7.0
Microtubule	0.6	2	30	11	5.4	43.0

Figure 7.2

Electrophoresis of fractions obtained in the hexylene glycol method for the purification of microtubules

The fractions were prepared as described in the legend to Table 7.1. Samples of each (containing approximately 100 μg of protein) were loaded on to the gels and electrophoresis was performed using phosphate-SDS buffer (as described in Chapter 2). The gels were fixed and stained with Coomassie brilliant blue. Results of CB-assays on the fractions are given in Table 7.1.



supernatant → gradient microtubule
soluble

given in Table 7.2, showing that the fractions below the 0.8M sucrose layer were enriched in CB-activity (R.S.A. 1.2-1.9). About one-third of the recovered CB-activity was found in the 0.8M-1.3M fraction.

Results of the electrophoresis of these samples are given in Fig. 7.3, showing that a major band was obtained (corresponding to a molecular weight of 60,000) in the 1.3M-1.5M fraction, together with several other fainter protein bands. In the 1.8M-2.0M fraction, however, there was a much greater purification of the 60,000 molecular weight protein band. This was the fraction in which mainly bundles of microtubules were reported to be found.

Zinc ions are also reported to aggregate and precipitate CB-protein. This was tested by incubating purified CB-protein (see Chapter 6) with 1mM $ZnCl_2$ and 1mM GTP at 37° for 1h. The sample became turbid after a short time, indicating that protein was being aggregated in the solution. The mixture was centrifuged, and the precipitate obtained was assayed for CB-activity. It was found that practically all the CB-activity originally present had been precipitated by this method. When repeated using a whole supernatant from brain, similar results were obtained. Results of electrophoresis of the Zn^{2+} -derived samples are given in Fig. 7.4, and show that the precipitated material was enriched in the 60,000 molecular weight protein band, although it also contained many other proteins.

Table 7.2

CB-activity of microtubule preparations made by
the Zn²⁺-GTP method

Rat brain was homogenised in 10mM imidazole-HCl buffer, pH 7.2, containing 1mM ZnCl₂ and 1mM GTP at room temperature. The homogenate was layered over a gradient consisting of 6ml each of 2.0M, 1.8M, 1.5M, 1.3M and 0.8M sucrose in homogenising medium, and was centrifuged at 13,000g for 30 min. A photograph of the gradient obtained is shown in Figure 7.1. Samples collected from each of the interfaces were dialysed against 10mM phosphate-Mg²⁺ buffer, and were assayed for CB-activity. The results given were obtained using 1g of brain in the preparation. Results of electrophoresis of the fractions are given in Figure 7.2.

Table 7.2

CB-activity of microtubule preparations made by the Zn²⁺-GTP method

<u>Fraction</u>	<u>Total protein(mg)</u>	<u>% recovered protein</u>	<u>CB-activity³ (bound cpm x 10³)</u>	<u>% recovered CB-activity</u>	<u>R.S.A.</u>	<u>S.A.³ (x 10³)</u>
0M sucrose	14.95	25	70	7	0.3	5
0M-0.8M interface	14.15	24	135	14	0.6	10
0.8M-1.3M interface	11.20	19	320	32	1.7	28
1.3M-1.5M interface	6.00	10	180	18	1.9	30
1.5M-1.8M interface	2.40	4	60	6	1.5	24
1.8M-2.0M interface	5.00	8	100	10	1.2	20
2.0M sucrose	6.70	11	130	13	1.2	19

Figure 7.3

Electrophoresis of fractions obtained in the Zn^{2+} -GTP method for the purification of microtubules

The fractions were prepared as described in the legend to Figure 7.1. Approximately 100 μ g of each sample was loaded on to each gel and electrophoresis was performed, using phosphate-SDS buffer (see Chapter 2). The gels were fixed and stained with Coomassie brilliant blue. The CB-activities of the fractions are given in Table 7.2.

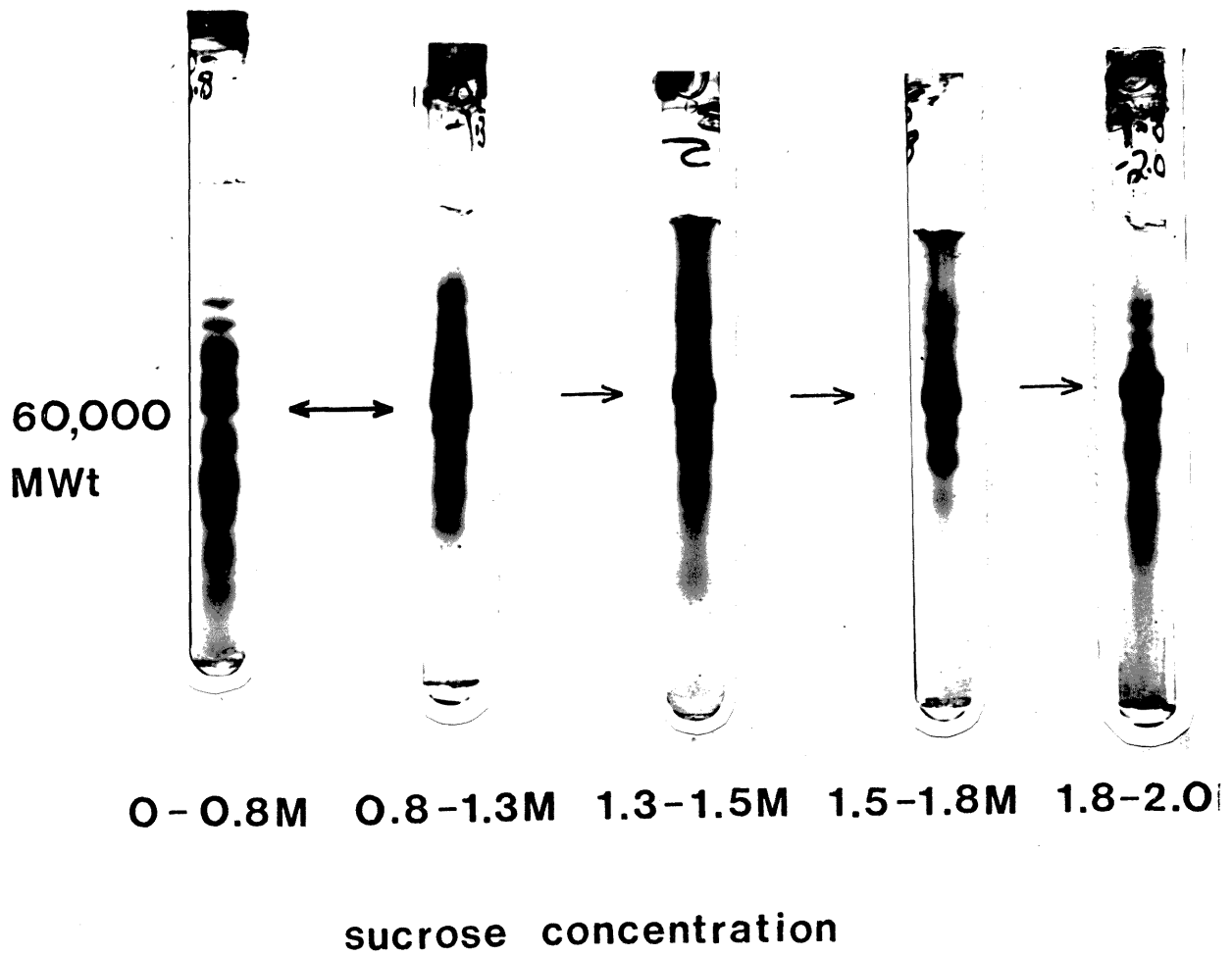
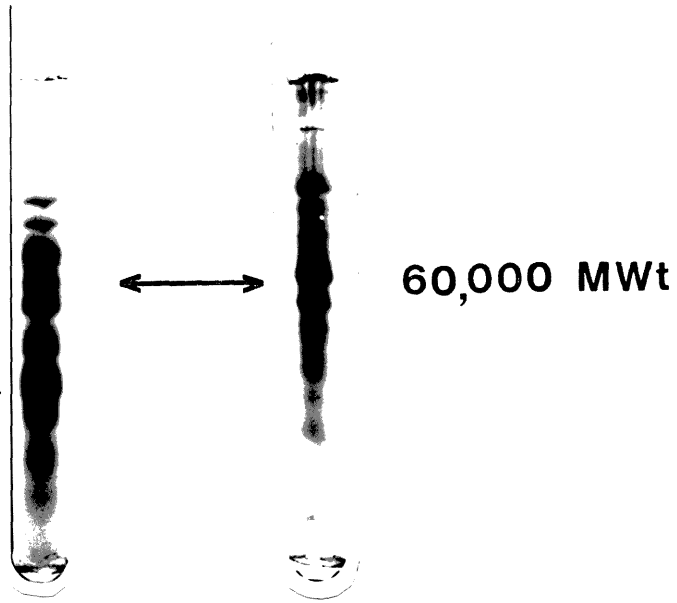


Figure 7.4

Electrophoresis of Zn²⁺-precipitated material from
soluble extracts of rat brain

A rat brain soluble extract was incubated with 1mM ZnCl₂ and 1mM GTP for 1h. at 37^o. The mixture was centrifuged and the fractions obtained were electrophoresed (applying approximately 100 µg of protein/gel) using phosphate-SDS buffer (see Chapter 2). The gels were fixed and stained with Coomassie brilliant blue.



supernatant

pellet

CHAPTER EIGHT

Discussion

Is microtubule protein (tubulin) colchicine-binding protein?

It has not been successfully established to date that colchicine-binding is a property of intact microtubules or microtubular protein from brain, but this has been assumed on the basis of circumstantial evidence. It is known that addition of colchicine to cells results in the disappearance of microtubular structures; under conditions of depolymerisation of microtubules (e.g. disruption of cells in cold buffered sucrose solutions), a protein can be recovered from the soluble fraction which binds colchicine. This protein is assumed to be derived from microtubules. Direct proof of this assumption required that intact microtubules be isolated (in a microtubule-stabilising medium) and purified, and tested for their ability to bind colchicine. The finding that the fraction reported to be rich in microtubules was greatly enriched in CB-activity (see Chapter 7) was the first evidence that colchicine-binding was a property of brain microtubular protein. The earlier failures of other workers to detect colchicine-binding in brain microtubule preparations was due to the interference of the stabilising medium with the CB-assay; this was overcome in these experiments by simple dialysis of the preparations in the cold. It was first shown (Shelanski and Taylor, 1967) that microtubule preparations obtained from sea-urchin sperm tails contained colchicine-binding protein; however, the starting material was essentially a pure source of microtubules, and did not require the use of a stabilising medium in the purification.

Additional evidence has more recently appeared connecting CB-protein with brain microtubules. This involves the isolation, in GTP-containing media, of soluble CB-protein from mammalian brain, and its polymerisation into tubular structures when incubated at 37°. The purified structures could be purified by centrifugation, then depolymerised by cooling, in repeating cycles (Weisenberg, 1972; Borisy and Olmsted, 1972; Olmsted and Borisy, 1973; Shelanski et al, 1973). The depolymerised protein, at the end of each cycle, was found to be more highly purified on the basis of CB-activity and electrophoresis.

Results from both these approaches thus clearly indicate an important connection between microtubular protein and CB-protein in brain.

What is the relation between soluble brain tubulin and tubulin from other sources?

Analysis of CB-protein purified from mammalian brain (see Chapter 6) showed it to have very similar properties to microtubule subunit protein obtained from various other sources; for example on the basis of its molecular weight (from electrophoretic data), amino acid composition, ability to bind colchicine, sugar content and enzymic properties. There appears to exist, therefore, a well-defined class of closely related proteins which are the subunits of at least some of the microtubules seen by electron microscopy.

The kinetic parameters and binding constant for soluble brain tubulin (see Chapter 5) were found to agree well with those obtained using different preparations. Maximum binding occurred after 2 to 3h. at 37^o, as had been found for tubulin extracted from sea-urchin eggs and from KB cells (Borisy and Taylor, 1967 (a,b)) and from grasshopper embryos (Wilson and Friedkin, 1967). The binding constant for the reaction, found to be between 2 μM and 3 μM , agreed well with that found for sea-urchin egg tubulin (2.3 μM) and KB cell tubulin (4 μM) (Borisy and Taylor, 1967 (a,b)). The optimal pH for the binding reaction agreed with the value of 6.8 found for tubulin extracted from chick embryo brain (Wilson, 1970).

Binding of colchicine to tubulin has been found to be a reversible process, which is time and temperature dependent. This was first demonstrated by Borisy and Taylor (1967 (a)), who allowed the soluble fraction of HeLa cells to stand for varying times at 0^o prior to the CB-assay. They found that after about 4h. ageing of the preparation, only approximately half the original CB-activity measured was found to remain. However, ageing of the preformed protein-colchicine complex under these conditions showed very little loss of bound material. A similar loss in CB-activity was seen in microtubule protein isolated from pig brain (Weisenberg et al, 1968), and from chick embryo brain (Wilson, 1970) in which the rate of the decay process was increased using a temperature of 37^o. Under these conditions, the losses of binding activity in the unbound and pre-bound samples were very similar. It appears probable, therefore, that the experimental conditions do not directly influence the formation of the protein-colchicine complex, but affect the CB-reaction indirectly by inactivating the protein at a faster rate. The pattern of results obtained by Wilson (1970) were confirmed when rat brain soluble preparations were aged at 37^o (see Chapter 5);

the observation in these experiments that the protein sample became turbid as the incubation proceeded supports the idea that the protein undergoes denaturation, or aggregation to a non-colchicine-binding form, while the loss in measured CB-activity is occurring. A striking requirement for sucrose was found necessary to stabilise CB-activity in frozen samples (see Chapter 5). This effect was also later confirmed (Frigon and Lee, 1972), although the function of the sucrose in this instance is not known.

CB-protein purified from pig brain was found to contain 0.5-1.0 mole of bound GTP per 120,000g when isolated in GTP-free buffer (Weisenberg et al, 1968); it was also found to bind 2 moles of GTP, one of which was freely exchangeable. Addition of GTP to a solution of the protein also considerably stabilised the CB-activity during storage at 0°; this effect was also seen at 37° (see Chapter 5), although to a lesser extent. The initial CB-activity of the preparations was higher in the presence of GTP and increased as the GTP concentration was increased; also the decay rate was slowed. This suggests that GTP and colchicine do not interact with CB-protein at the same site. The influence of the binding of GTP could therefore be considered as an allosteric effect, possibly altering the conformation of the protein; this could modify its ability to function as a subunit of microtubules.

The effects of the Vinca alkaloids vinblastine and vincristine (which, like colchicine, are antimitotic agents and disrupt microtubule structure) on the CB-activity of tubulin-containing preparations were investigated, since earlier work had shown that vinblastine stimulated CB-activity in grasshopper embryos (Wilson and Friedkin, 1967). This work also indicated that the colchicine-binding site of the protein was different to the site of action of these drugs. The stabilising effects of vinblastine on CB-activity in chick embryo brain extracts (Wilson, 1970) were also seen with vincristine in rat brain extracts (see Chapter 5). However, it was additionally found that low concentrations of vincristine (10^{-5} to 10^{-6} M), in the cold, produced a redistribution of CB-activity in soluble extracts, actually almost specifically precipitating tubulin. This had not previously been observed, and was apparently dependent on the presence of Mg^{2+} ions in the preparation. Indeed, a concentration of 1mM $MgCl_2$ or higher was found to be necessary for this effect to occur. Much higher concentrations of vinblastine (10^{-3} M) had been previously used at 37° to precipitate tubulin from various cells (Marantz et al, 1969; Olmsted et al, 1970; Wilson et al, 1970), although these protein preparations were

far from pure, and were seen to contain both high and low molecular-weight impurities (e.g. actin) after electrophoresis, showing that these alkaloids could not be used as very specific reagents for this purpose. It is interesting that in other attempts to purify microtubular protein, based on a succession of polymerisation-depolymerisation reactions (Borisy et al, 1974), similar contaminating proteins were also co-purified. It is not yet known whether these are proteins specifically associated with microtubules, or are other species that reversibly aggregate under conditions promoting microtubule assembly. The high molecular-weight material has also been observed in hexylene glycol preparations of intact microtubules (Kirkpatrick et al, 1970).

What could be the functions of brain microtubules?

Some investigators have speculated that microtubules extend continuously along neuronal processes to provide the directional motive force for the cytoplasmic transport of vesicles and other particulate matter (Schmitt, 1968; Schmitt and Samson, 1968). Colchicine and the Vinca alkaloids, vinblastine and vincristine, are now often used as probes for microtubule-associated functions in various cell types, since it is known that agents which disrupt microtubules decrease or block axoplasmic transport (Junqueira and Porter, 1969; Fernandez et al, 1970; Moran and Varela, 1971); hence the effects of these drugs on neural function could be related to their ability to bind tubulin in one form or another.

The finding that purified brain CB-protein could serve as a substrate for an 'intrinsic' protein kinase, which was closely associated with the protein (Goodman *et al*, 1970), was confirmed and extended to other species (pig and rabbit), and a variety of assay conditions (altering the pH, time of incubation, and composition of the assay mixture). Phosphorylation of the protein was found to be linear with time during the first 10 minutes, and this activity was linear with respect to protein concentration, showing that tubulin was the main substrate for its associated kinase activity. Phosphorylation of the protein was found to increase in the presence of added cAMP; in addition, it was observed that both the α - and β -forms of tubulin were phosphorylated, to approximately the same extent, when the labelled protein was electrophoresed in urea-containing buffer. Moreover, in spite of the relatively small stimulation of the intrinsic kinase activity obtained with cAMP, it was found that the purified CB-protein bound between 4-22 pmoles of the cyclic nucleotide/mg of protein when assays were conducted at 4^o. It is conceivable that this cAMP-stimulated phosphorylation reaction could be involved in controlling the assembly and function of microtubular proteins in various cell types (cf. Gillespie, 1971; Hsie and Puck, 1971).

The relation of the protein kinase associated with isolated tubulin preparations to other protein kinases present in soluble extracts of brain is not clear. The possibility that the tubulin intrinsic kinase activity may be a property of the tubulin itself, and not of an associated enzyme, cannot be excluded, since virtually all the evidence attempting to demonstrate that the kinase and its substrate (tubulin) are distinct proteins (see Eipper, 1974) is not conclusive. The experiments performed involve the use of added exogenous (basic) protein substrates to measure the activity of the tubulin-associated kinase; however, the nature of the protein substrate(s) phosphorylated was not specified. It is therefore not known whether or not the increase in kinase activity observed in the presence of added basic proteins is due to an increase in enzyme activity toward the tubulin substrate.

Some difficulties arose in trying to determine the relation of the tubulin-associated kinase to its protein substrate, when the effects of Mn²⁺ and of the Vinca alkaloids and colchicine on the tubulin intrinsic kinase activity were compared with those measured in the presence of added basic proteins as substrates. Mn²⁺ had been found (Miyamoto *et al*, 1969) to stimulate soluble histone kinase activity of brain, whereas this was not found to be so for the tubulin intrinsic kinase. Also, in the

presence of colchicine, or vincristine or vinblastine (at concentrations similar to those which produce selective aggregation of tubulin), there was a marked increase in the amounts of ^{32}P transferred to tubulin in the presence of cAMP, negligible effects of these drugs occurring in its absence. Electrophoretic separation of the labelled protein samples followed by radioautography confirmed that tubulin was the main protein substrate phosphorylated. However, colchicine or the Vinca alkaloids did not increase the activity of tubulin-associated kinases when assayed with histones as substrates, or that of a histone kinase prepared from rat brain by the method of Miyamoto.

In view of the preferential effects of the Vinca alkaloids and colchicine on the cAMP-stimulated intrinsic kinase activity, the possible effects of these drugs on the binding of cAMP to tubulin preparations was tested, but the only effect observed was a small inhibition of binding. It would appear, therefore, that the effects of these antimitotic agents on the intrinsic kinase activity of brain tubulin are fairly specific, and could possibly be related to the effect of the drugs on the state of aggregation of the tubulin. This has been indicated in recent work in which tubulin has been phosphorylated in situ, and has been recovered in the form of high molecular weight aggregates (Reddington and Lagnado, 1973; Eipper, 1974).

A potentially important recent observation (Lagnado et al, 1975) has also indicated that the membrane-bound tubulin present in synaptic complexes can be phosphorylated in vitro by a cAMP-stimulated protein kinase present in the synaptic membranes. This finding could prove to be of major importance in our understanding of the role of membrane-bound tubulin.

The observation that microtubular proteins exhibit intrinsic protein kinase activity suggests that this enzyme might be responsible for phosphorylating tubulin in situ. Further, the stimulation of this protein kinase activity by cAMP supports the possibility that microtubular structure and/or function could be controlled by phosphorylation mechanisms regulated by changes in cyclic nucleotide metabolism. The fact that tubulin is a major component of nerve cells also suggests that the phosphorylation of tubulin might be of fundamental importance in the control of some aspects of neural function.

What are the structures responsible for particulate colchicine-binding in brain?

One of the main observations emerging from the work presented in this thesis is that in brain, as compared to other tissues investigated, a substantial proportion of the colchicine-binding activity remains firmly bound to particulate fractions, and cannot be removed by washing the preparations. During the initial part of this work, it was shown that about half the measured CB-activity present in homogenates of rat, rabbit and guinea-pig brain was recovered in the crude particulate fraction obtained after high-speed centrifugation. Subfractionation of this crude particulate preparation showed that most of the CB-activity recovered in the crude nuclear fraction was due to trapped soluble material, while the synaptosome fraction obtained from crude mitochondrial preparations accounted for almost half the CB-activity recovered from the parent fraction. Also, a large proportion of the CB-activity (30%) recovered from the crude mitochondrial fraction was found in the synaptic-membrane fraction. These results were in keeping with those of Feit and Barondes (1970), who found, during the course of this work, that in mouse brain about 50% of the homogenate CB-activity was recovered in the crude particulate fraction and was unequally distributed amongst the various subfractions of the crude mitochondrial preparation. A small percentage of the particulate CB-activity could be attributed to entrapped soluble material (possibly of synaptosomal origin?), as was found in the present work: about 20% of the activity present in the crude mitochondrial preparation could be solubilised after osmotic lysis under conditions known to release synaptosomal cytoplasm and/or material that is loosely bound to membranes (see Chapter 4, and Fig. 4.11). However, only about 20% of the remainder of the membrane-bound CB-activity could be extracted by treatment with Triton X-100 (see Chapter 5, and Table 5.3), suggesting that the CB-protein was closely associated with, or was an integral component of, synaptic junctional material. An additional interesting finding was that the synaptic vesicle fraction prepared also showed CB-activity, although it accounted for a much smaller proportion of the total activity recovered.

It was assumed that in all the preparations tested, high-affinity binding proteins were involved, which showed similar CB-characteristics. This assumption was later shown to be valid (see Chapter 5, and below in this Discussion). The decay rate of the colchicine-binding process has recently

been shown to increase with dilution of the protein solution (Bamburg et al, 1973); it must therefore be a possibility that maximal binding was not measured in each of the preparations, and this could have produced some small errors in the calculation of relative specific CB-activities.

Electrophoresis of the subcellular fractions showed the presence of a tubulin-comigrating protein in each of the fractions, including the synaptosome-soluble sample, indicating that tubulin, and possibly microtubules, could be present in the cytoplasm of nerve terminals, as well as being components associated with synaptic junctional membranes. The presence of microtubules in the synaptic region has now been demonstrated in subcellular work (Gray, 1975). Using a new fixation technique, it was shown that microtubules run from the axon and contact the dense projections of the pre-synaptic cleft region. This arrangement could presumably allow vesicles, which are seen associated with the microtubules, to be guided to their site of action.

The work on synaptic preparations presented in this thesis provided the first clear-cut evidence that they are enriched in a tubulin-like protein, as defined on the basis of CB-properties. Particulate 'tubulin' was found to be associated with some kind of special membrane found in the region of the synapse. In retrospect, it has now been shown (Tan, 1975; Walters and Matus, 1975), on the basis of electrophoretic data, that a tubulin-like protein is a structural component of synaptic junctional complexes. This was achieved by extracting purified synaptosomal membranes overnight with 1.2% sodium deoxycholate, yielding, after high-speed centrifugation, a pellet which contained the post-synaptic junctional densities. Electrophoresis of the samples on polyacrylamide gels in SDS-buffer showed that the detergent-insoluble material had been enriched in a protein which comigrated with purified tubulin. Additionally, when the sample was electrophoresed in SDS- and urea-containing gels, this band could be split into the characteristic tubulin doublet. The preparation showed CB-activity, but no microtubules were apparent when examined electron-microscopically.

It is therefore apparent that a tubulin-like protein is tightly bound in synaptosomal membranes, and is thus probably present in the junctional structures themselves. This would explain both the occurrence of tubulin in the preparations obtained, and its great enrichment in the isolated junctional structures.

Other workers (Bhattacharyya and Wolff, 1975) have also succeeded in solubilising CB-protein from isolated synaptic membranes using the detergent Nonidet P-40, which released 16% of the membrane-bound protein and up to 90% of the CB-activity. Electrophoresis of the solubilised protein showed that tubulin-comigrating protein was one of the main components of the preparation. These results, taken together, would oppose the view held by Bamberg et al, (1973) that the microtubule protein associated with the particulate fraction 'merely results from entrapment of the soluble neurotubule protein in vesicles formed from the myelinated axons'. This situation was compared to the formation of vesicles containing ribosomes during homogenisation of brain (Gambetti et al, 1972); the explanation probably arose due to the inclusion, in their preparative procedure, of sonication which has been shown (see Chapter 5) to redistribute a proportion of soluble brain tubulin to the particulate fraction when followed by centrifugation. However, since sonication is not normally included in the preparation of synaptic membranes, this phenomenon is presumably not involved.

Does the amount or distribution of tubulin change with development of the brain?

The discovery that extracts of immature mammalian brain had greater specific CB-activities than those measured in adult tissue extracts was of interest. This difference could be connected with the higher turnover rate of cell components in immature cells, due to the increased growth rate (hence an increased CB-protein content), or alternatively, due to the dilution of nerve cells, in the adult, with non-nerve cells (i.e. glial cells).

However, the results could also indicate that CB-protein from immature brain is present in a more stabilised form than in the adult (i.e. having a slower CB-activity decay rate) giving an apparently higher CB-activity, compared to the adult. This could have been determined by comparing the effects of ageing at 37° on extracts derived from immature tissue with those found for adult extracts (see Chapter 5, and Fig. 5.8). However, the results reported are consistent with the findings of Bamberg et al, (1973), who later compared the properties of soluble extracts of immature and adult chick brain. They found that the CB-activity decay rate was slowest in immature brain extracts, but it rapidly increased as development continued. This indicated that a change was occurring in the CB-protein during development, and it was suggested that microtubule proteins with different functions could be involved in the development of the brain.

The shift in distribution of protein and CB-activity from the soluble to the particulate fraction observed during development could reflect the changes occurring during the differentiation of neuronal cells, when synaptic junctions are formed, since it was found that synaptic membranes contribute a substantial proportion of the particulate CB-activity (see Chapter 4). The highest CB-activities in chick brain (Bamburg *et al*, 1973) were measured between 9 and 28 days of incubation (i.e. 6 days after hatching). This age range corresponds neurologically to that of 'immature' (1-day old) rat brain and 'foetal' (28-day, i.e. full-term) rabbit brain used in the experiments on developing brain. The differences in distribution with development could possibly be due to differences in the binding properties of the immature-derived preparations as compared to those obtained from adult brain, although the binding characteristics of the adult soluble and particulate preparations had been found to be very similar in many respects (see Chapter 5). The decrease in specific CB-activity in the soluble extract during development could be due to a dilution by the more rapid synthesis of other, non-colchicine-binding proteins. These observed changes in distribution of CB-activity have since been extended by the very recent finding of Jones (1976) that tubulin reaches its adult level in developing synaptic membranes earlier than other synaptic membrane proteins (these observations being based on quantitative estimations from electrophoretic protein separations). In addition, this indicates that tubulin may be involved in establishing the basic framework of the junction.

An obvious question arising from the results concerns the relationship of the CB-sites associated with nerve-membrane components (particularly synaptic membranes) with CB-proteins of presumed microtubular origin which are present in the soluble fraction. Most of the evidence that soluble CB-protein purified from nerve tissue represents microtubule subunit protein is of an indirect nature; the significance of membrane-bound CB-protein in relation to microtubule structure and function also still remains to be elucidated.

Does particulate CB-protein resemble soluble tubulin?

The kinetics of the colchicine-binding reaction were found to be very similar when preparations of soluble, particulate and membrane-bound CB-proteins were compared (see Chapter 5), indicating that the same class of macromolecule was responsible for the colchicine-binding in each case.

One important line of future investigation emerging from these findings would concern the specificity of the binding reaction - how similar are the soluble and particulate binding sites? Or how specific is the colchicine in its binding requirements?

The membrane components which bind colchicine appear to represent stabilised forms of microtubular protein, since their CB-activity is largely retained after storage of the membranes at -10° for up to two weeks, or after repeated washings with phosphate-buffered sucrose solutions containing 0.1% Triton X-100 (see Chapter 5). In fact, it was found necessary to use the detergent SDS (concentration 1%) for solubilisation (which was non-specific) of the membrane-bound CB-protein. Dialysis of the SDS out of the preparation was found to be necessary due to its inhibitory effect on colchicine-binding; this step was not carried out in more recent work (Bhattacharyya and Wolff, 1975), consequently CB-activity was not detected in their preparations. These properties contrast with the more rapid decay of CB-activity observed in soluble preparations under similar conditions. The comparative stability of the tightly-bound particulate and membrane CB-protein could be explained by considering that other constituents of the membranes may afford structural protection; alternatively, that the membranes could contain components which stabilise the CB-protein, these components being absent, or present to a smaller extent, in the soluble preparation.

In these respects, the work of Bhattacharyya and Wolff (1975) is of particular interest. They report that membrane-bound CB-protein, in contrast to soluble tubulin from rat brain, was relatively stable when subjected to thermal inactivation. However, when the CB-protein was solubilised from the membranes (using the detergent Nonidet P-40, which did not inhibit colchicine-binding), it decayed at the same rate as did soluble tubulin. This indicates that the increased stability of the membrane-bound tubulin is not an intrinsic property of the protein itself, but rather of the environment of the membrane.

These results could suggest that the interruption of various physiological processes in nerve tissue by colchicine may be partly due to the binding of this drug by nerve-ending membranes, in addition to any direct interactions it may have with microtubular proteins. This could involve either the relatively loosely-bound (Triton-extractable) form (which is possibly presynaptic), or the more tightly-bound tubulin (which is probably present in the post-synaptic complex), which could also be concerned with direct action at the nerve-ending itself. The latter would appear a more likely

possible explanation of the mechanism of action involved. The idea that tubulin in the nerve-endings may organise material resulting in the transmission of stimuli needed evidence that tubulin exists in synaptic junction preparations. This was established (electrophoretically) in the work of Walters and Matus (1975), and (immunohistochemically) in the work of Matus et al, (1975) which showed that all the anti-tubulin staining in brain tissue was associated with either microtubules or the post-synaptic membrane. No visible evidence of microtubule-like ultra-structure was obtained within the post-synaptic lattice, suggesting that the tubulin present is not self-polymerised as it is in microtubules, but is bound to other proteins of the lattice structure. The synaptosomal membranes showed CB-activity, so obviously were composed of a colchicine-binding form of tubulin; could this be the residue of disrupted remnants of microtubules incorporated in the membranes? If this were the case, a link would be provided between the junctional region and the microtubular system of the dendrite.

Recent peptide mapping of tryptic digests of tubulin and tubulin-comigrating synaptosomal protein (Blitz and Fine, 1974; Kornguth and Sunderland, 1975; Walters and Matus, 1975) has indicated that the proteins are identical; moreover, it has also been demonstrated that the major polypeptide component of isolated postsynaptic junctional lattices (solubilised by deoxycholate treatment) possesses the same tryptic peptide map as that of purified tubulin (Walters and Matus, 1975). A striking homology was thus found between the proteins (of approximate Mwt 50,000) extracted from synaptosomal plasma membranes, postsynaptic junctional lattices and purified brain tubulin, which were so closely similar in primary structure that their tryptic digests were virtually identical. This information appears to confirm the identification of tubulin as the major component of the postsynaptic density. One could now speculate that junctional tubulin could be involved in two methods of molecular organisation:

- (a) binding to other molecular components of the junctional lattice, and
- (b) providing a terminus for microtubules communicating with the dendritic system.

Many questions still remain to be answered concerning the mechanisms and control of the microtubule assembly process. The role of calcium ion in the polymerisation process is not clear, and it is not known how the binding and splitting of GTP is related to microtubule assembly. The chemical nature of the nucleation centres involved in tubule polymerisation has not

been determined, nor has their actual function, if any. The detailed function and mechanism of action of microtubules in chromosome movement, intracellular motility, and generation of cell form is virtually unknown. Recent advances on the in vitro assembly of microtubules may provide approaches by which many of these questions relating to in vivo control could be investigated.

REFERENCES

- 1 ADELMAN, M R, BORISY, G G, SHELANSKI, M L, WEISENBERG, R C and TAYLOR, E W (1968) Fed. Proc. 27, 1186.
- 2 BALAZS, R and COCKS, W (1967) J. Neurochem. 14, 1035.
- 3 BAMBURG, J R, SHOOTER, E M, and WILSON, L (1973) Biochem. 12, 1476.
- 4 BEHNKE, O and ZELANDER, T (1967) J. Ultrastruct. Res. 19, 147.
- 5 BENSCH, K G and MALAWISTA, S E (1969) J. Cell Biol. 40, 95.
- 6 BENSCH, K G, MARANTZ, R, WISNIEWSKI, H and SHELANSKI, M (1969) Science 165, 495.
- 7 BHATTACHARYYA, B and WOLFF, J (1975) J. Biol. Chem. 250, 7639.
- 8 BORISY, G G and OLMSTED, J B (1972) Science 177, 1196.
- 9 BORISY, G G, OLMSTED, J B, MARCUM, J M and ALLEN C (1974) Fed. Proc. 33, 167.
- 10 BORISY, G G and TAYLOR, E W (1967,a) J. Cell Biol. 34, 525.
- 11 BORISY, G G and TAYLOR, E W (1967,b) J. Cell Biol. 34, 535.
- 12 BRADFIELD, J R G (1955) S.E.B. Symposia IX p306.
- 13 BRAY, G A (1960) Anal. Biochem. 1, 279.
- 14 BRYAN, J (1971) J. Exptl. Cell Res. 66, 129.
- 15 BRYAN, J and WILSON, L (1971) Proc. Natl. Acad. Sci. (US) 68, 1762.
- 16 BURTON, K (1956) Biochem. J. 62, 315.
- 17 DAHL, D R, REDBURN, D A and SAMSON, F E Jr (1970) J. Neurochem. 17, 1215.
- 18 DAHLSTROM, A (1968) Eur. J. Pharm. 5, 111.
- 19 DAVISON, P (1970) Advanc. Biochem. Psychopharm. 2, 289.
- 20 DUTTON, G R and BARONDES, S (1969) Science 166, 1637.
- 21 EIPPER, B A (1972) Proc. Natl. Acad. Sci. (US) 69, 2283.
- 22 EIPPER, B A (1974) J. Biol. Chem. 249, 1398.
- 23 FAIRBANKS, G, LEVINTHAL, C and TEEDER, R H (1965) Biochem. Biophys. Res. Comm. 20, 393.
- 24 FALXA, M L and GILL, T J (1969) Arch. Biochem. Biophys. 135, 194.
- 25 FEIT, H and BARONDES, S H (1970) J. Neurochem. 17, 1355.

- 26 FERNANDEZ, H, BURTON, P and SAMPSON, F (1971) J. Cell Biol. 51, 176.
- 27 FERNANDEZ, H, HUNEEUS, F and DAIVSON, P (1970) J. Neurobiol. 1
- 28 FRIGON, R P and LEE, J C (1972) Arch. Biochem. Biophys. 153, 587.
- 29 GALL, J G (1966) J. Cell Biol. 31, 639.
- 30 GAMBETTI, P, AUTILLO-GAMBETTI, A, GONATES, N K and SHAFER, B (1972) J. Cell Biol. 52, 526.
- 31 GIBBONS, I (1963) Proc. Natl. Acad. Sci. (US) 50, 1002.
- 32 GIBBONS, I and GIBBONS, B (1972) J. Cell Biol. 54, 75.
- 33 GIBBONS, I and GRIMSTONE, A (1960) J. Biophys. Biochem. Cytol. 7, 697.
- 34 GIBBONS, I and RENAUD, (1968) J. Cell Biol. 36, 79.
- 35 GILLESPIE, E (1971) J. Cell Biol. 50, 544.
- 36 GOODMAN, D, RASSMUSSEN, H, DiBELLA and GUTHROW, C (1970) Proc. Natl. Acad. Sci. (US) 67, 652.
- 37 GOODWIN, T W and MORTON, R A (1946) Biochem J. 40, 628.
- 38 GORDON, M and BARNETT, R J (1967) Exptl. Cell Rs. 48, 395.
- 39 GRAFSTEIN, B, McEWEN, B S and SHELANSKI, M (1970) Nature 227, 289.
- 40 GRAY, E G (1975) Proc. Roy. Soc. B. 190, 369.
- 41 GROSSFELD, R M and SHOOTER, E M (1971) J. Neurochem. 18, 2265.
- 42 HAVSTEEN, B H (1966) J. Theor. Biol. 10, 1.
- 43 HSIE, A and PUCK, T (1971) Proc. Natl. Acad. Sci. (US) 68, 358.
- 44 INOUÉ, S (1952) Exptl. Cell Res. 2, 305.
- 45 INOUÉ, S and SATO, H (1967) J. Gen. Physiol. 50, Suppl. 259.
- 46 JAROSH, R (1964) Primitive Motile Systems in Cell Biology, Acad. Press Inc., New York, p599.
- 47 JONES, D H (1976) Thesis, London University.
- 48 JUNQUEIRA, L C and PORTER, K R
- 49 KANE, R E (1965) J. Cell Biol. 25, 137.
- 50 KATAOKA, K and de ROBERTIS, E (1967) J. Pharmacol. Exptl. Therap. 156, 114.
- 51 KATZ, N L (1972) Eur. J. Pharmacol. 19, 88.
- 52 KIRKPATRICK, J (1969) J. Cell Biol. 42, 600.
- 53 KIRKPATRICK, J, HYAMS, L, THOMAS, V and HOWLEY, P (1970) J. Cell Biol. 47, 384.

- 54 KREUTZBERG, G (1969) Proc. Natl. Acad. Sci (US) 62, 722.
- 55 KUO, J and GREENGARD, P (1969) J. Biol. Chem. 244, 3417.
- 56 LAEMMLI, U (1970) Nature 227, 680.
- 57 LAGNADO, J, TAN, L P and REDDINGTON, M (1975) Annals N Y Acad. Sci. 253, 577.
- 58 LAPETINA, E, SOTO, E and de ROBERTIS, E (1967) Biochem. Biophys. Acta 135, 33.
- 59 LOWRY, O H, ROSEBROUGH, N J, FARR, A L, and RANDALL, R J (1951) J. Biol. Chem. 193, 265.
- 60 LUDUENA, R and WOODWARD, D O (1972) Fed. Proc. 31, 882.
- 61 MARANTZ, R and SHELANSKI, M (1970) J. Cell Biol. 44, 234.
- 62 MARANTZ, R, VENTILLA, M and SHELANSKI, M (1969) Science 165, 498.
- 63 MARCHALONIS, and WELTMAN, (1971) J. Comp. Bioch. Physiol. 38, 609.
- 64 MARGOLIS, R and SHELANSKI, M (1972) Biochem. Biophys. Res. Comm. 47, 432.
- 65 MAZIA, D (1955) S.E.B. Symposia IX, p335.
- 66 McGUICKIN, W F and McKENZIE, B F (1958) Clin. Chem. 4, 476.
- 67 McINTOSH, J R, HEPLER, P K and VAN WIE, D G (1969) Nature 224, 659.
- 68 MIYAMOTO, E, KUO, J and GREENGARD, P (1969) J. Biol. Chem. 244, 6395.
- 69 MIZEL, S B and WILSON, L (1972) Biochem. 11, 2572.
- 70 MORAN, and VARELA, (1971) Proc. Natl. Acad. Sci. (US) 68, 757.
- 71 COTMAN, C W and MATTHEWS, D A (1971) Biochem. Biophys. Acta 249, 380
- 72 OLMSTED, J and BORISY, G (1973) Biochem. 12, 4282.
- 73 OLMSTED, J B, CARLSON, K, KLEBE, R, RUDDLE, F and ROSENBAUM, J (1970) Proc. Natl. Acad. Sci. (US) 65, 129.
- 74 PERISIC, M and CUÉNOD, M (1972) Science 175, 1140.
- 75 PETERS, A and VAUGHN, (1967) J. Cell Biol. 32, 113.
- 76 PETERS, A, PALAY, S and WEBSTER, H (1970) The Fine Structure the Nervous System, New York: Harper, p198.
- 77 PORTER, K R (1966) CIBA Foundation Symposia on Principles of Biomolecular Organisation, Churchill Ltd, London, p308.
- 78 PORTER, K R (1966) Principles of Biomolecular Organisation, ed. G E WOLSTENHOLME and M O'CONNOR, Boston; Little, Brown.
- 79 PRASAD, K and HSIE, A (1971) Nature New Biol. 233, 141.

- 80 PROCEEDINGS of the BECKMANN SUMMER SCHOOL (1967)
- 81 REDDINGTON, M and LAGNADO, J (1973) Fed. Eur. Bioch. Soc. Lett. 30, 188.
- 82 RENAUD, F, ROWE, A and GIBBONS, I (1966) J. Cell Biol. 31, 92A.
- 83 RINGO, D (1967) J. Ultrastruct. Res. 17, 266.
- 84 SABATINI, D, BENSCH, K and BARNETT, R (1963) J. Cell Biol. 17, 19.
- 85 SCHMITT, F (1968) Proc. Natl. Acad. Sci. (US) 60, 1005.
- 86 SCHMITT, F and SAMSON, F (1968) Neurosci. Res. Prog. Bull. 6, 113.
- 87 SHELANSKI, M, GASKIN, F and CANTOR, C (1973) Proc. Natl. Acad. Sci. (US) 70, 765.
- 88 SHELANSKI, M and TAYLOR, E (1967) J. Cell Biol. 34, 549.
- 89 SHELANSKI, M and TAYLOR, E (1968) J. Cell Biol. 38, 304.
- 90 SJOSTRAND, J, FRIZELL, M and HASSELGREN, P (1970) J. Neurochem. 17, 1563.
- 91 SOUTHERN RESEARCH INSTITUTE (1971) (February) p154.
- 92 STEPHENS, R E (1968) J. Mol. Biol. 32, 277.
- 93 STEPHENS, R E (1970) J. Mol. Biol. 47, 353.
- 94 STEPHENS, R E and LEVINE, E E (1970) J. Cell Biol. 46, 416.
- 95 TAN, L P (1975) PhD Thesis, London University.
- 96 TAO, M (1971) Arch. Biochem. Biophys. 143, 151.
- 97 TAO, M, SALAS, M and LIPMANN, F (1970) Proc. Natl. Acad. Sci. (US) 67, 408.
- 98 TAO, M and LIPMANN, F (1969) Proc. Natl. Acad. Sci. (US) 63, 86.
- 99 WALTERS, B and MATUS, A (1975) Biochem. Soc. Trans. 3, 109.
- 100 WATTS, D C and BANNISTER, L H (1970) Nature 226, 450.
- 101 WEBER, K and OSBORN, M (1969) J. Biol. Chem. 244, 4406.
- 102 WEISENBERG, R (1968) PhD Thesis, University of Chicago.
- 103 WEISENBERG, R (1972) Science 177, 1104.
- 104 WEISENBERG, R, BORISY, G and TAYLOR, E (1968) Biochem. 7, 4466.
- 105 WEISENBERG, R and TIMASHEFF, S (1970) Biochem. 9, 4110.
- 106 WEURKER, R and KIRKPATRICK, J (1972) Intl. Rev. Cytol. 33, 45.
- 107 WHITTAKER, V (1964) Biochem. J. 90, 273.
- 108 WHITTAKER, V and SHERIDAN, M (1965) J. Neurochem. 12, 363.

- 109 WILSON, L (1970) Biochem. 9, 4999.
- 110 WILSON, L, BRYAN, J, RUBY, A and MASIA, D (1970) Proc. Natl. Acad. Sci. (US) 66, 807.
- 111 WILSON, L and FRIEDKIN, M (1966) Biochem. 5, 2463.
- 112 WILSON, L and FRIEDKIN, M (1967) Biochem. 6, 3126.
- 113 WILSON, L and FRIEDKIN, M (1968) Biochem.
- 114 WITMAN, G, CARLSON, K, BERLINER, J and ROSENBAUM, J (1968) J. Cell Biol. 36, 79.
- 115 YANAGISAWA, T, HASIGAWA, S and MOHRI, H (1968) Exptl. Cell Res. 52, 86.
- 116 ZACHARIUS, R M, ZELL, T E, MORRISON, J H and WOODLOCK, JJJ (1969) Anal. Biochem. 30, 148.

Some Properties of Colchicine-Binding Protein(s) ('Microtubule Protein') in Soluble and Particulate Preparations of Mammalian Brain

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The kinetic properties and stability of colchicine-binding proteins present in soluble extracts of brain have been extensively studied (Borisy & Taylor, 1967a,b; Weisenberg *et al.*, 1968; Wilson & Friedkin, 1968; Wilson, 1970). In contrast, little is known about the properties of membrane-bound colchicine-binding activity, which accounts for a considerable proportion of that present in homogenates of immature and adults brain (Lagnado *et al.*, 1971a,b; see also Feit & Barondes, 1970).

Further work shows that Mg^{2+} (final concn. 1-10mM) markedly enhance colchicine-binding activity in both soluble and particulate fractions derived from rat brain homogenates prepared in PMS buffer (0.32M-sucrose in 1mM-sodium phosphate buffer, pH 6.5) after high-speed centrifugation. However, the colchicine-binding activity of both soluble and particulate fractions prepared in 20mM-phosphate buffer, pH 6.8, containing 100mM-sodium glutamate was less than half that seen in PMS buffer, even though the stabilizing effects of the phosphate-glutamate buffer seen after storage of the preparations at $-10^{\circ}C$ (cf. Wilson, 1970) was confirmed. The protective effects of GTP on colchicine-binding activity was especially evident for the soluble fraction in the absence of added Mg^{2+} .

The kinetic properties and stability of colchicine-binding activity of samples prepared in PMS buffer were studied at various temperatures (between 2° and $55^{\circ}C$). Maximum values for colchicine-binding activity were obtained after incubation for 1.5-2.5 h at all temperatures tested for both soluble and particulate preparations, highest values occurring at $37^{\circ}C$. However, stability studies showed that the half-life of colchicine-binding activity for particulate preparations was at least twice that seen for soluble preparations at several temperatures. It was also found that preincubation of both soluble and

particulate preparations at $1^{\circ}C$ (before assay at $37^{\circ}C$) was accompanied by an initial increase of colchicine-binding activity, reaching a maximum of about 200% of control values at 3 h.

Relatively low concentrations (1-50 μM , final) of vinblastine or vincristine stabilize colchicine-binding activity in soluble extracts of chick-embryo brain (Wilson, 1970). Similar results are found for both the soluble and particulate preparations of rat, guinea-pig and rabbit brain. Moreover, inclusion of these alkaloids (at a final concn. of 50 μM) in the initial homogenizing medium (PMS buffer) caused a remarkable redistribution of colchicine-binding activity after high-speed centrifugation: about 95% of the activity was recovered in the particulate fraction, as compared with values of 30-45% in the absence of alkaloid. This phenomenon was not seen in the absence of added Mg^{2+} and was not due to the preferential activation of the particulate colchicine-binding activity. Indeed, the addition of vincristine (50 μM) to a high-speed supernatant containing Mg^{2+} also induced the precipitation of colchicine-binding activity after recentrifugation.

The present results, together with additional observations on the properties of membrane-bound colchicine-binding proteins, are consistent with the view that particulate colchicine-binding activity may represent a stabilized form of microtubule protein (Lagnado *et al.*, 1971b). The possible significance of these findings in relation to the role of microtubules in nerve tissue will be discussed.

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- Borisy, G. & Taylor, E. W. (1967a) *J. Cell Biol.* **34**, 525
Borisy, G. & Taylor, E. W. (1967b) *J. Cell Biol.* **34**, 535
Feit, H. & Barondes, S. (1970) *J. Neurochem.* **17**, 1355
Lagnado, J. R., Lyons, C. A. & Wickremasinghe, G. (1971a) *Biochem. J.* **122**, 56 p
Lagnado, J. R., Lyons, C. A. & Wickremasinghe, G. (1971b) *FEBS Lett.* **15**, 254
Weisenberg, R., Borisy, G. & Taylor, E. W. (1968) *Biochemistry* **7**, 4466
Wilson, L. (1970) *Biochemistry* **9**, 4999
Wilson, L. & Friedkin, M. (1968) *Biochemistry* **6**, 3126

**The Possible Significance of Adenosine
3':5'-Cyclic Monophosphate-Stimulated
Protein Kinase Activity Associated with
Purified Microtubular Protein Preparations
from Mammalian Brain**

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It has been reported that cyclic AMP (adenosine 3':5'-cyclic monophosphate) stimulates the phosphorylation of microtubular protein (i.e. colchicine-binding) purified from soluble extracts of ox brain by a protein phosphokinase closely associated with the purified protein (Goodman *et al.*, 1970). These observations have now been confirmed and extended for microtubular proteins purified from soluble extracts of pig, rabbit and rat brain. Protein kinase activity was usually assayed by an adaptation of the filter-disc method of Tao *et al.* (1970), or by the procedure of Weller & Rodnight (1971) by measuring ³²P in protein-bound phosphoserine residues.

Most of the ³²P incorporated into microtubular protein, after incubation with [γ -³²P]ATP in the presence or in the absence of added cyclic AMP (final concn. 50 μ M), was present in the major band (mol.wt. 58000) seen after electrophoresis of incubated samples in polyacrylamide gels according to the method of Weber & Osborn (1969). However, electrophoresis in tris-buffered gels containing sodium dodecyl sulphate and urea, which results in the separation of two main forms of 'tubulin' monomer (see Bryan & Wilson, 1971; Feit *et al.*, 1971; Olmsted *et al.*, 1971), indicates that the faster component appears to be preferentially labelled.

Colchicine and vincristine, which are known to bind to microtubular proteins, did not show consistent effects, although in general vincristine stimulated kinase activity in the absence of added cyclic AMP. Mn²⁺ (final concn. 100 μ M) markedly inhibits microtubular kinase activity, which thus resembles the 'intrinsic' kinase of synaptosome

membranes (Weller & Rodnight, 1971), but differs from the soluble histone kinase studied by Miyamoto *et al.* (1969).

Microtubular protein preparations from pig and rabbit brain also bind cyclic AMP, as determined by the method of Tao *et al.* (1970) with cyclic [³H]-AMP. Maximum values of 1-4 mmol of bound cyclic [³H]AMP/mol of 'tubulin' monomer (mol.wt. 58000) were obtained. Under similar conditions ³²P incorporation into serine residues seldom exceeds 20 mmol/mol of the monomer. The extent to which these relatively low values represent exchange reactions remains to be determined.

Dibutyryl cyclic AMP (6-N,2'-O-dibutyryl-adenosine 3':5'-cyclic monophosphate) (final concn. 100 μ M) markedly stimulates the growth of neurites in chick-embryo sympathetic-ganglia cultures grown in the absence of added nerve growth factor. The growth-promoting effects of both cyclic AMP and nerve growth factor are abolished by low concentrations of drugs known to prevent microtubule assembly (see also Prasad & Hsie, 1971). The possibility that cyclic AMP might stimulate neurite growth through the phosphorylation of free or membrane-bound (see Lagnado *et al.*, 1971) microtubule subunits, and indeed that nerve growth factor may act partly through stimulation of adenylate cyclase activity, will be discussed in the light of experiments still in progress.

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- Bryan, J. & Wilson, L. (1971) *Proc. Nat. Acad. Sci. U.S.* 68, 1762
Feit, H., Slusarek, L. & Shelanski, M. (1971) *Proc. Nat. Acad. Sci. U.S.* 68, 2028
Goodman, D., Rasmussen, H., DiBella, F. & Guthrow, C. (1970) *Proc. Nat. Acad. Sci. U.S.* 67, 652
Lagnado, J., Lyons, C. & Wickremasinghe, G. (1971) *FEBS Lett.* 15, 254
Miyamoto, E., Kuo, J. & Greengard, P. (1969) *J. Biol. Chem.* 244, 6395
Olmsted, J., Witman, G., Karlson, K. & Rosenbaum, J. (1971) *Proc. Nat. Acad. Sci. U.S.* 68, 2273
Prasad, K. & Hsie, A. (1971) *Nature New Biol. (London)* 233, 141
Tao, M., Salas, M. & Lipmann, F. (1970) *Proc. Nat. Acad. Sci. U.S.* 67, 408
Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* 244, 4406
Weller, M. & Rodnight, R. (1971) *Biochem. J.* 124, 393

The Subcellular Distribution of Colchicine-Binding Protein(s) ('Microtubule Protein') in Rat Brain

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The ability of colchicine and other antimitotic alkaloids (e.g. vinblastine) to disrupt cytoplasmic microtubules in brain and other tissues appears to parallel the abundance, in these tissues, of a soluble protein that binds colchicine (Adelman, Borisy, Shelanski, Weisenberg & Taylor, 1968; Wilson & Friedkin, 1968). Much indirect evidence (for review see Schmitt & Samson, 1968) suggests that the colchicine-binding protein present in soluble extracts of brain and other tissues represents the main subunit of microtubules and that the interruption of several physiological processes in neural tissue by antimitotic alkaloids such as colchicine may be due to their interaction with microtubule protein.

More recently it was observed that at least 50% of the colchicine-binding activity in brain homogenates is associated with the crude particulate fraction obtained after high-speed centrifugation (see, e.g., Dahl, Redburn & Samson, 1970), although no intact microtubular structures could be detected in such preparations (J. R. Lagnado & C. Lyons, unpublished work). The subcellular distribution of colchicine-binding activity was therefore further investigated by differential and sucrose-gradient centrifugation of rat cerebral-cortex homogenates prepared in 0.32M-sucrose containing 1mM-phosphate-Mg²⁺ buffer, pH 6.5. Colchicine-binding activity was assayed in the various fractions by the filter-disc method of Weisenberg, Borisy & Taylor (1968) as described by Wilson (1970) with [³H]colchicine (specific radioactivity 1.7 Ci/mmol).

It was found that about 45% of the recovered radioactivity is present in the crude nuclear (approx. 32%) and mitochondrial (approx. 13%) fractions. The relative specific radioactivities (% of radioactivity recovered/% of protein recovered)

for these fractions ranged between 0.6 and 0.8, in contrast with the values found for the microsomal (1.6-2.4) and soluble fractions (1.5-2.8), which accounted for approx. 8 and 47% of the total recovered radioactivity. Most of the radioactivity present in crude nuclear preparations was due to non-nuclear contamination. Nerve-ending particles ('synaptosomes') purified from washed mitochondrial preparations by the procedure of Gray & Whittaker (1962) accounted for nearly 50% of the radioactivity present in the primary fraction and exhibited relative specific radioactivities several times those of purified mitochondria. Further, it was found that subfractionation of water-lysed nerve-ending-particle preparations by the procedure of Whittaker & Sheridan (1965) released about 15% of colchicine-binding activity in soluble form, while about 10, 30 and 40% of the remaining activity was associated with the vesicle ('D₁'), small-membrane ('D₂') and residue ('J') fractions. The highest relative specific radioactivities (>2) were shown by fractions D₁ and D₂.

The possible relation of membrane-bound and soluble colchicine-binding protein(s) to microtubule protein will be discussed. Some of the results obtained are comparable with those reported for mouse brain by Feit & Barondes (1970).

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- Adelman, M., Borisy, G., Shelanski, M., Weisenberg, R. & Taylor, E. W. (1968). *Fedn Proc. Fedn Am. Soc. exp. Biol.* **27**, 1186.
Dahl, R., Redburn, D. & Samson, F. E., jun. (1970). *J. Neurochem.* **17**, 1215.
Feit, H. & Barondes, S. (1970). *J. Neurochem.* **17**, 1355.
Gray, E. G. & Whittaker, V. P. (1962). *J. Anat.* **96**, 79.
Schmitt, F. O. & Samson, F. E., jun. (1968). *Neurosci. Res. Program Bul.* **6**, 113.
Weisenberg, R., Borisy, G. & Taylor, E. W. (1968). *Biochemistry, Easton*, **7**, 4466.
Whittaker, V. P. & Sheridan, M. (1965). *J. Neurochem.* **12**, 363.
Wilson, L. (1970). *Biochemistry, Easton*, **9**, 4999.
Wilson, L. & Friedkin, M. (1968). *Biochemistry, Easton*, **6**, 3126.

THE SUBCELLULAR DISTRIBUTION OF COLCHICINE-BINDING PROTEIN ('MICROTUBULE PROTEIN') IN RAT BRAIN

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1. Introduction

The disruption of cytoplasmic microtubules in brain and other tissues by colchicine and other anti-mitotic alkaloids (e.g. vinblastine) is generally supposed to result from a direct interaction of these drugs with microtubule protein [1, 2]. Circumstantial evidence indicates that the colchicine-binding protein purified from soluble extracts of neural tissue represents the major subunit (MW 120,000) of microtubules. It was recently shown, however, that more than 50 percent of the colchicine-binding activity in buffered sucrose homogenates of brain is associated with the crude particulate fraction obtained after high-speed centrifugation [3-5], although no intact microtubules can be detected under these conditions [6].

The nature of the particulate components responsible for colchicine-binding was therefore investigated after subcellular fractionation of rat brain cortex homogenates by differential and gradient centrifugation. The results demonstrate that the fractions enriched in nerve-ending particles ('synaptosomes') represent a major site for colchicine-binding activity, most of which is associated with the purified synaptic membrane fraction.

2. Material and methods

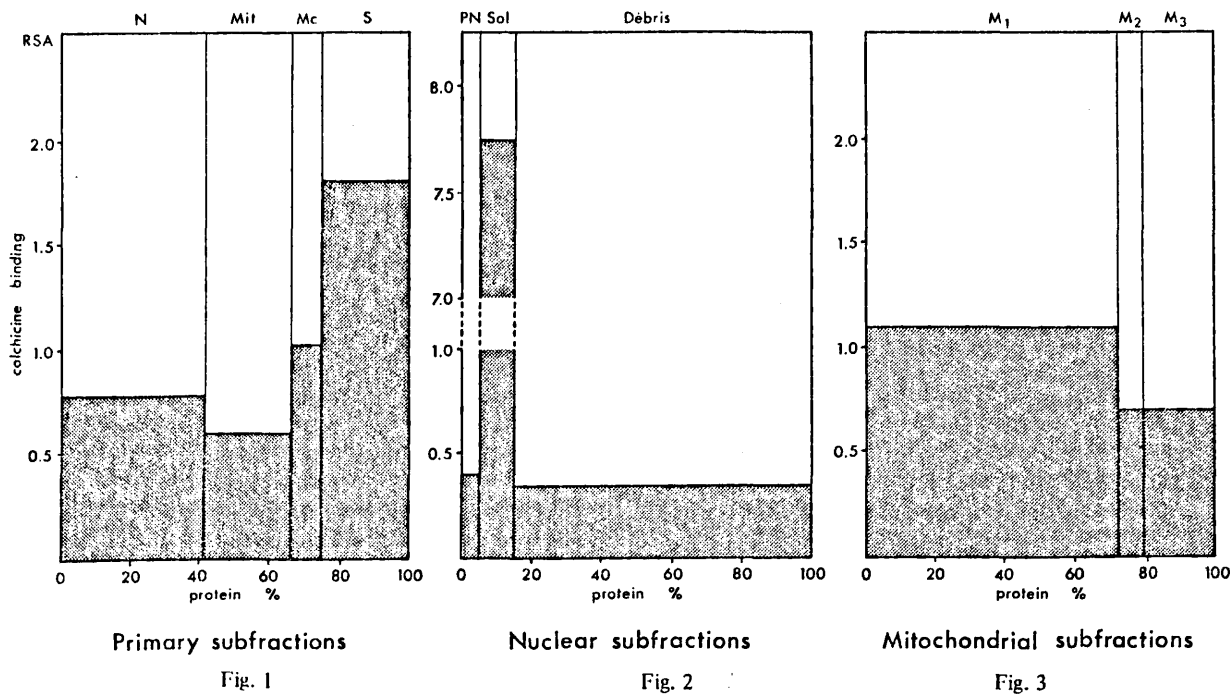
2.1. Subcellular fractionation

Rat brain cortex was homogenised in 9 parts of 0.32 M sucrose containing 1 mM sodium phosphate-Mg²⁺ buffer, pH 6.5. Primary fractions (crude nuclear,

mitochondrial, microsomal, soluble) and purified nuclei were prepared according to the method of Balazs and Cocks [7]. The purification of nerve-ending membranes (i.e. synaptic membranes) and vesicles from osmotically disrupted mitochondrial suspensions was carried out following the procedure of Lapetina et al. [8], as summarized in table 1, and the homogeneity of the subfractions was checked by electron microscopy. The purity of the nuclear fraction was checked by light microscopy and DNA determinations [9]. All fractions to be assayed were resuspended in, or adjusted to, 10 mM sodium phosphate-Mg²⁺ buffer, pH 6.5, and kept on ice (maximum 3 hr) prior to incubation.

2.2. Assay of colchicine-binding activity

Bound ³H-colchicine was assayed by the filter-disc (DE81) method of Weisenberg et al. [10], as modified by Wilson [11], except that incubations were carried out in 10 mM sodium phosphate-Mg²⁺ buffer pH 6.5, instead of in phosphate-glutamate buffer [11]. The different subcellular fractions were diluted prior to assay to give a protein concentration of 100-1000 µg/ml reaction mixture, in which range colchicine-binding was found, in preliminary experiments, to be proportional to protein concentration. Protein-bound ³H-colchicine absorbed on DE81 filter discs was counted directly in 5 ml Bray's solution [12] in a Packard 3375 spectrometer, at 48% efficiency. Radioactivity measurements were carried out on triplicate samples, and results given are based on the means of at least three separate experiments (maximum variability between experiments ± 10%).



Figs. 1-3. Diagram showing the colchicine-binding activity in relative specific activity (RSA) in relation to percentage of protein for the primary fractions, the nuclear Subfractions after sucrose density centrifugation and the mitochondrial subfractions after osmotic shock. The relative specific activity is defined in Methods. The content of the mitochondrial subfractions is given in table 1.

³H-Colchicine (specific radioactivity 1.7 Ci/mmol) was obtained from the Radiochemical Centre, Amersham. Protein concentration was determined by the method of Lowry et al. [13].

2.3. Expression of results

Colchicine-binding activities are expressed as Relative Specific Activities (RSA's), in order to facilitate comparison of colchicine-binding activity in the various subfractions with that of the parent fraction, whose RSA is taken to be 1.

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

The recoveries for proteins and colchicine-binding activity were not less than 90% and 75%, in that order, throughout the subcellular fractionation procedures.

3. Results

3.1. Distribution of colchicine-binding (CB) activity in primary fractions

After differential centrifugation of rat brain homogenates prepared in buffered sucrose (see section 2.1), 46 ± 5.4% (n = 8) of the CB-activity was found in the soluble fraction, while the crude nuclear, mitochondrial and microsomal fractions accounted for 31 ± 2.6 (5), 15 ± 2.7 (5) and 8 ± 1.7 (5) percent, in that order, of the activity recovered. Taking the protein distribution into account, it is apparent from the data shown in fig. 1 that there was a nearly two-fold enrichment of CB-activity in the soluble fraction (RSA 1.88 ± 0.28 (8)), while the RSA values for the particulate fractions did not exceed 1.0.

The crude nuclear fraction was further purified by centrifugation through 1.67 M sucrose [7], yielding 3 subfractions: a purified nuclear pellet (PN), an intermediate soluble phase (Sol) and a floating

Table 1
Colchicine-binding activity of brain mitochondrial subfractions.

Subfractions	Conditions	Description	Colchicine-binding activity (%)
<i>(A) Subfractions of crude mitochondria (Mit) after osmotic shock</i>			
M ₁	20,000 g × 30 min pellet	myelin, mitochondria, synaptic membranes	80
M ₂ + M ₃	20,000 g × 30 min sup't	synaptic vesicles, membranes, soluble	20
M ₂	100,000 g × 60 min pellet	synaptic vesicles, some membranes	5
M ₃	100,000 g × 60 min sup't	soluble	15
<i>(B) Subfractions of M₁ after sucrose gradient centrifugation, 50,000 g for 120 min</i>			
M ₁ 0.8	see figs. 3 and 4	myelin	12
M ₁ 0.9		synaptic membranes, some myelin	18
M ₁ 1.0		synaptic membranes	21
M ₁ 1.2		synaptic membranes	27
M ₁ (P)		mitochondria, some membranes	22
<i>(C) Subfractions of M₂ after sucrose gradient centrifugation, 50,000 g for 60 min</i>			
M ₂ A	0.32–0.5 M sucrose	synaptic vesicles	78
M ₂ B	0.5 M sucrose	membranes, some synaptic vesicles	22

Crude mitochondrial preparations were derived from rat brain cortex, and subfractionated after osmotic shock according to the method of Lapetina et al. [8]. Colchicine-binding activity was assayed as specified in section 2.2 and values for individual subfractions, representing the means of at least 3 experiments, are given as a percentage of the activity recovered from the parent fraction.

debris layer which were found to contain 2, 80 and 18 per cent, in that order, of the activity recovered from the parent fraction. The data shown in fig. 2 clearly demonstrate that brain nuclei obtained by this procedure represent a minor source of CB-activity. These findings were confirmed using crude nuclear preparations derived from new-born rat brain which, however, contained only 2% of the homogenate CB-activity, as compared with values of about 30% found for adult brain – (see above). The higher activity seen with adult brain can be more readily understood if one considers that myelin, which represents a minor constituent in immature brain, is largely responsible for the trapping of both soluble and particulate contaminants found in the

crude nuclear fraction [7]. Similarly, it is possible that the relatively high concentration of CB-activity found in the crude microsomal fraction (fig. 1) is due, in part at least, to the presence of small nerve-endings known to contaminate this fraction [14].

The possibility that CB-activity in the crude mitochondrial fraction was due to non-mitochondrial constituents (e.g., nerve-ending particles) was therefore investigated.

3.2. Mitochondrial subfractions (table 1)

It was found that the bulk of the activity present in crude mitochondrial suspensions disrupted by osmotic shock was recovered in the pellet obtained after low-speed centrifugation (table 1 A, subfraction

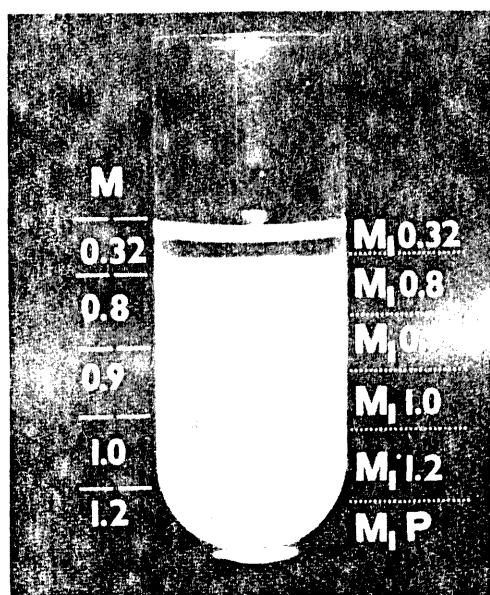


Fig. 4. Photograph of the gradient of M_1 , made according to the technique of Lapetina et al. [8], showing the sucrose concentrations and the various layers and pellet which were separated. The content of the various subfractions and their colchicine-binding activities are given in table 1 and fig. 3.

M_1). Most of the remaining activity was found in the soluble fraction (M_3) obtained after further centrifugation at high speed, which represents mainly the soluble constituents of nerve-ending particles. Fig. 3 shows that subfraction M_1 was a richer source of CB-activity than either M_2 or M_3 .

Further fractionation of M_1 by sucrose gradient centrifugation (fig. 4) resulted in a considerable enrichment of CB-activity in those fractions which contain predominantly synaptic membranes (M_1 0.9, M_1 1.0 and M_1 1.2, fig. 5), though this was not apparent if the distribution of proteins was not taken into account (table 1 B). Taken together, the fractions rich in synaptic membranes account for about 70% of the CB-activity of M_1 , or over half of the activity originally present in the crude mitochondrial fraction. This represents a considerable degree of localization of CB-protein in synaptic membranes. Similar results were obtained in experiments using 1-day old rat brain and with highly purified synaptic membrane preparation

obtained from beef brain (unpublished observations of Lagnado and Lyons).

Finally, it was shown that most of the CB-activity present in M_2 was associated with the synaptic vesicle fraction ($M_2 A$) purified by an additional sucrose gradient centrifugation step (table 1 C, fig. 6).

4. Discussion

An obvious question arising from the results described concerns the relationship of the colchicine-binding sites associated with nerve membrane components (especially synaptic membranes) with colchicine-binding proteins of presumed microtubular origin which are present in the soluble fraction. The possibility that membrane components which bind colchicine represent stabilized forms of microtubular protein is not inconsistent with the observation that colchicine-binding activity of synaptic membranes is largely retained after repeated washings with phosphate-buffered sucrose solutions containing 0.1% Triton X100, or after storage of the membranes at -10° for up to two weeks (Lyons and Lagnado, paper in preparation; cf. also [3, 4]). This is in contrast with the rapid decay of colchicine-binding activity observed with soluble preparations under similar conditions [10].

The possible significance of membrane-bound CB-protein in relation to microtubule structure and function remains a matter for speculation, especially since to our knowledge, most of the evidence that the soluble colchicine-binding protein purified from nerve tissue represents microtubule subunit protein is of indirect nature [2, 6]. However, it is perhaps worth noting that extensively purified colchicine-binding protein prepared from soluble extracts of pig brain possesses both an intrinsic cyclic AMP-activated phosphokinase activity [see also 15], as well as the capacity to bind cyclic AMP, as measured by the methods of Tao et al. [16] (Lagnado, Lyons and Weller, paper in preparation). In many respects, the properties of the phosphokinase and cyclic AMP-binding activities present in these preparations were found to be similar to those originally described for purified synaptic membranes from beef brain [17].

In conclusion, it is suggested that the interruption

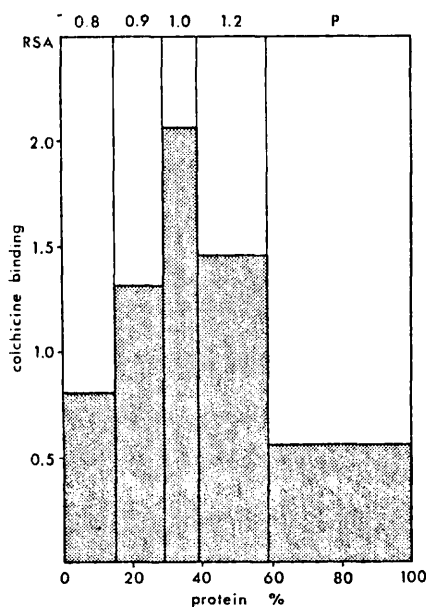
Subfractions of M₁

Fig. 5

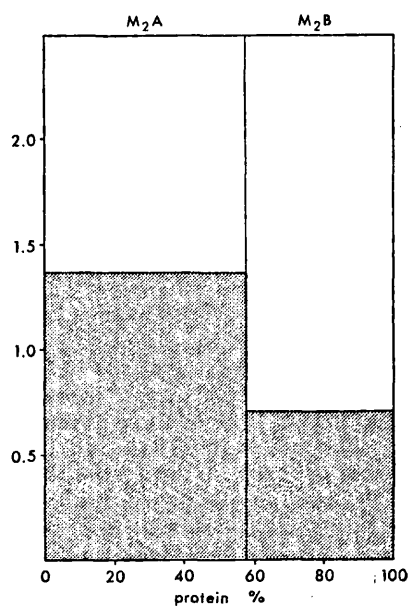
Subfractions of M₂

Fig. 6

Figs. 5 and 6. Diagram showing the colchicine-binding activity in relative specific activity (RSA) in relation to the percentage of protein for the subfractions of M₁ and the subfractions of M₂ after sucrose density centrifugation. The relative specific activity is defined in Methods. The content of the M₁- and M₂-subfractions is given in table 1. The density gradient obtained from fraction M₁ is shown in fig. 4.

of various physiological processes in nerve tissue by colchicine and related alkaloids [1, 2] may be partly due to the binding of these drugs by nerve-ending membranes in addition to any direct interactions they may have with microtubular proteins.

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References

- [1] F.O. Schmitt and F.E. Samson, Jun., *Neurosci. Res. Program Bul.* 6 (1968) 113.
 - [2] M. Adelman, B. Borisy, M. Shelanski, R. Weisenberg and E.W. Taylor, *Federation Proc.* 27 (1968) 1186.
 - [3] R. Dahl, D. Redburn and F.E. Samson, Jun., *J. Neurochem.* 17 (1970) 1215.
 - [4] H. Feit and S. Barondes, *J. Neurochem.* 17 (1970) 1355.
 - [5] J.R. Lagnado, C. Lyons and Wickremasinghe, *Biochem. J.* 122 (1971) in press.
 - [6] J. Kirkpatrick, L. Hyams, V. Thomas and P. Howley, *J. Cell Biol.* 47 (1970) 384.
 - [7] R. Balázs and W. Cocks, *J. Neurochem.* 14 (1967) 1035.
 - [8] E. Lapetina, E. Soto and E. De Robertis, *Biochim. Biophys. Acta* 135 (1967) 33.
 - [9] K. Burton, *Biochem. J.* 62 (1956) 315.
 - [10] R. Weisenberg, G. Borisy and E. Taylor, *Biochemistry, Easton* 7 (1968) 4466.
 - [11] L. Wilson, *Biochemistry, Easton* 9 (1970) 4999.
 - [12] G. Bray, *Analyt. Biochem.* 1 (1960) 279.
 - [13] O. Lowry, N. Rosebrough, A. Farr and R. Randall, *J. Biol. Chem.* 193 (1951) 265.
 - [14] K. Kataoka and E. De Robertis, *J. Pharmacol. Exptl. Therap.* 156 (1967) 114.
 - [15] M. Kinwano and D. Schlessinger, *Proc. Natl. Acad. Sci. U.S.A.* 66 (1970) 146.
 - [16] M. Tao, M. Salas and F. Lipmann, *Proc. Natl. Acad. Sci. U.S.A.* 67 (1970) 408.
 - [17] M. Weller and R. Rodnight, *Nature* 225 (1970) 187.
- (15) D.P. Goodman, H. Rasmussen, F. Dibella and C.B. Guthrow, Jr., *Proc. Natl. Acad. Sci. U.S.A.* 67 (1970) 652.