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

J.R. LAGNADO, C. LYONS and G. WICKREMASINGHE
Department of Biochemistry, Bedford College, Regent's Park, London, N.W. 1, England

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1. Introduction

The disruption of cytoplasmic microtubules in brain and other tissues by colchicine and other antimitotic 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-Mg^{2+} 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 homogenity 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-Mg^{2+} buffer, pH 6.5, and kept on ice (maximum 3 hr) prior to incubation.

2.2. *Assay of colchicine-binding activity*

Bound ^{3}H-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-Mg^{2+} 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 ^{3}H-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%).

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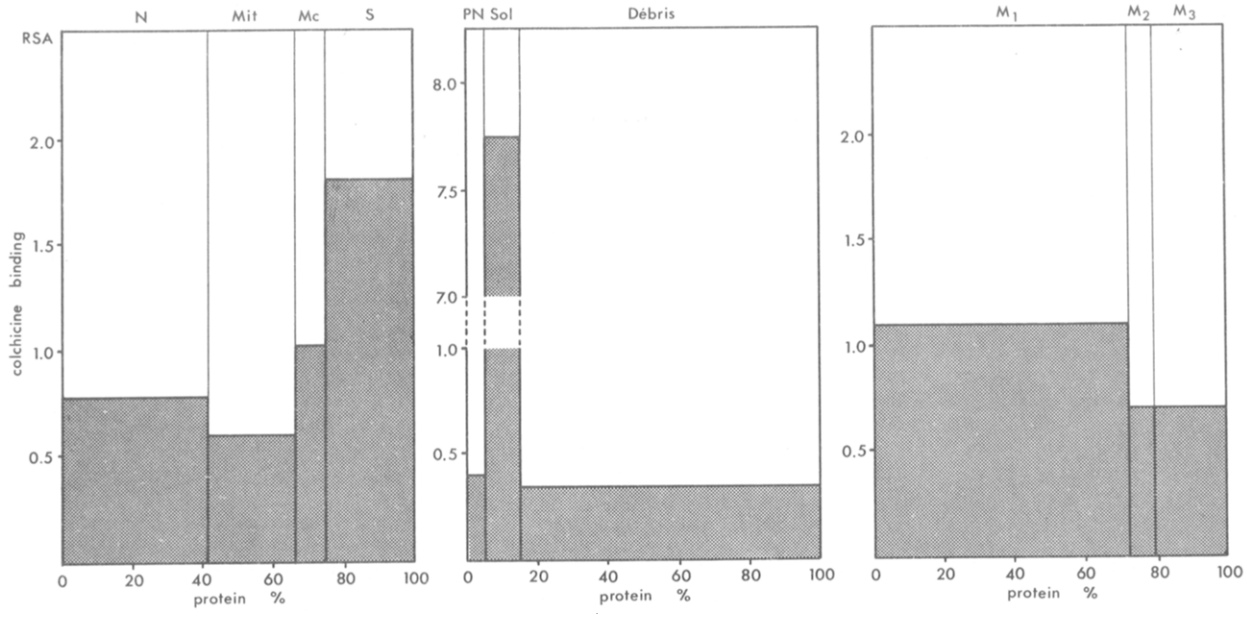


Primary subfractions

Fig. 1

Nuclear subfractions

Fig. 2

Mitochondrial subfractions

Fig. 3

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 defines in Methods. The content of the mitochondrial subfractions is given in table 1.

^{3}H-Colchicine (specific radioactivity 1.7 Ci/mmole) 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.

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

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 (%)
(A) *Subfractions of crude mitochondria (Mit) after osmotic shock*			
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
(B) *Subfractions of M_1 after sucrose gradient centrifugation, 50,000 g for 120 min*			
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
(C) *Subfractions of M_2 after sucrose gradient centrifugation, 50,000 g for 60 min*			
M_2A	0.32–0.5 M sucrose	synaptic vesicles	78
M_2B	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 percent, 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 on 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

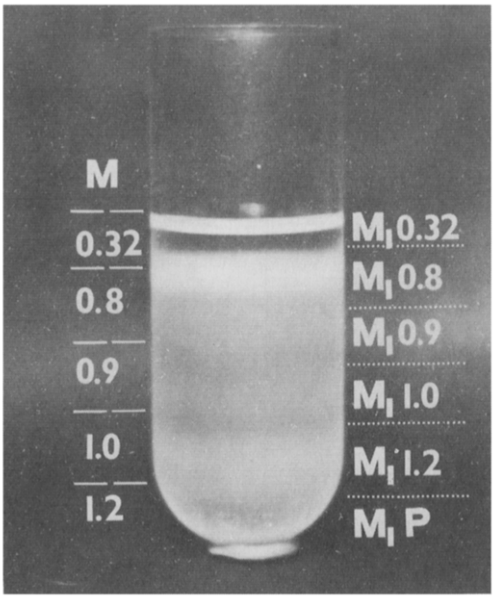


Fig. 4. Photograph of the gradient of M_1, made according to the technique of Lapetina et al. [8], showing the sucrose concentrations and the various layers and pellet which were separated. The content of the various subfractions and their colchicine-binding activities are given in table 1 and fig. 3.

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).

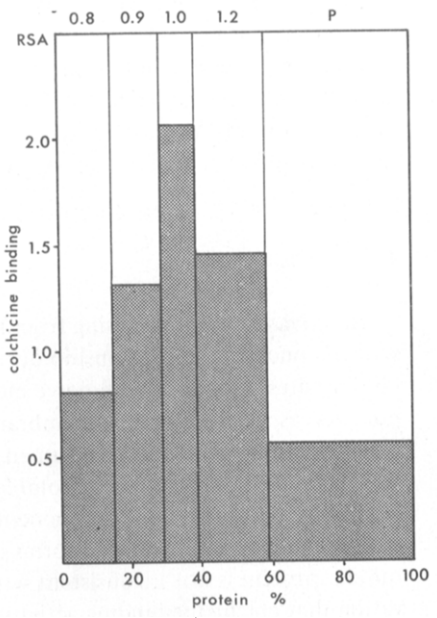
Finally, it was shown that most of the CB-activity present in M_2 was associated with the synaptic vesicle fraction (M_2 A) purified by an additional sucrose gradient centrifugation step (table 1 C, fig. 6).

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].

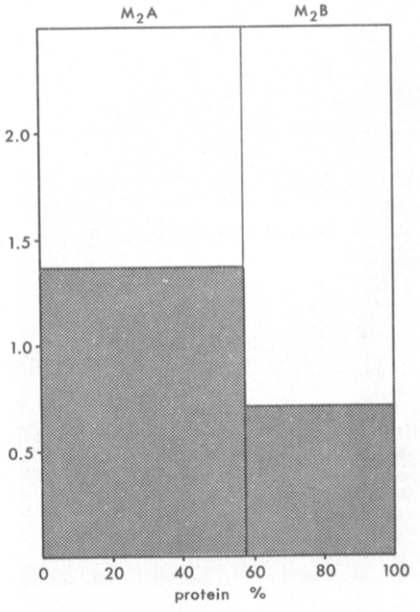
The possible significance of membrane-bound CB-protein in relation to microtubule structure and function remains a matter for speculation, especially since to our knowledge, most of the evidence that the soluble colchicine-binding protein purified from nerve tissue represents microtubule subunit protein 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 M_1

Fig. 5



Subfractions of M_2

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 M_1 and the subfractions of M_2 after sucrose density centrifugation. The relative specific activity is defined in Methods. The content of the M_1- and M_2-subfractions is given in table 1. The density gradient obtained from fraction M_1 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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