THE PHOSPHORYLATION OF COLCHICINE-BINDING ('MICROTUBULAR') PROTEIN IN RESPIRING SLICES OF GUINEA PIG CEREBRAL CORTEX

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1. Introduction

Adenosine 3′,5′-cyclic monophosphate (cyclic AMP) has been shown to influence the release of neurotransmitters at nerve endings [1] and the growth of neurites in cultured nerve tissue [2–4]; there is now some evidence that these processes depend on the integrity of cytoplasmic microtubules [2–5]. It is therefore conceivable that the cyclic nucleotide may interact with these processes through an effect on the structure and function of microtubules induced by a change in the state of phosphorylation of the tubules or their subunits. Indeed, it has recently been shown that the colchicine-binding (microtubular) protein derived from mammalian brain can act in the presence of [γ-^{32}P]ATP as a substrate for a cyclic AMP-stimulated protein kinase closely associated with the purified protein [3, 6]. Electrophoresis under disaggregating conditions further showed that most of the radioactivity in such preparations was associated with the faster-migrating of the two tubulin monomers separated (M.W. ca. 58,000) (see [3]). However, the extent to which microtubular proteins are phosphorylated in cell-containing preparations of nerve tissue remains to be established.

This matter has now been studied in experiments where respiring slices of guinea pig cerebral cortex were labelled during incubation with [^{32}P]orthophosphate. The results presented here indicate that the bulk of the protein-bound ^{32}P that is selectively precipitated with *Vinca* alkaloids from extracts of incubated tissue co-migrates with the purified tubulin monomer after electrophoresis in SDS-polyacrylamide gels, and that a significant proportion of the bound ^{32}P present in crude tubulin preparations obtained from incubated tissue by ammonium sulphate fractionation is associated with the colchicine-binding dimer detected by gel filtration and by sucrose-gradient centrifugation.

Materials and methods

2.1. *Materials*

Radioactive [^{32}P]orthophosphate and [^{3}H]colchicine were obtained from the Radiochemical Centre, Amersham, Bucks., U.K. Vincristinesulphate was from Eli Lilly Co. Ltd.

2.2. *Preparation and incubation of tissue*

Slices of guinea pig cerebral cortex were prepared from approx. 1 g tissue using a mechanical tissue chopper as described by McIlwain and Rodnight [7] and incubated in specially designed rapid release vessels (Reddington, unpublished) at 37° for 60 min in 40 ml of an oxygenated medium containing 128 mM NaCl, 6.3 mM KCl, 2.8 mM $CaCl_2$, 1.3 mM $MgCl_2$, 10 mM glucose, 25 mM Tris-HCl, pH 7.4 (at 37°C) and 1–1.5 mCi carrier-free [^{32}P]orthophosphate. Incubation was terminated by rinsing the slices with 500 ml ice-cold incubation medium and homogenising in the appropriate buffer (see below).

2.3. *Partial purification of tubulins from tissue incubated in vitro with ^{32}P*

2.3.1. Precipitation with vincristine (see [8])

Tissue was homogenised in 10 mM Tris-HCl, pH 7.4, to give a 20% (w/v) homogenate and centrifuged for 60 min at 100,000 g. Vincristine sulphate (VC) and $MgCl_2$ were added to the supernatant to give final conc. of 10^{-3} M and 2.5×10^{-3} M, respectively; after

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incubation at 37° for 10 min, the precipitate which formed was collected by centrifugation at 50,000 *g* for 20 min. In other experiments it had been shown that under these conditions VC precipitated 95–100% of the colchicine-binding activity of the high-speed supernatant. (M.R. unpublished data). Extraction of the VC precipitates prepared from ^{32}P-labelled material with 10% trichloroacetic acid (TCA) showed them to contain acid-insoluble radioactivity ($1-1.5 \times 10^4$ cpm/mg protein), approx. 50% of which was alkali-labile (see sect. 2.5).

The VC precipitate was washed twice with 10% TCA to remove adhering acid-soluble phosphates and further extracted with hot 5% TCA (90°, 15 min) or non-aqueous solvents (see sect. 3.2) to remove nucleic acids and lipids, respectively. The hot TCA extract contained approx. 40% of the total acid-insoluble counts in the VC pellet, whereas lipid extracts were unlabelled. The bulk of the labelled acid-insoluble phosphates of the VC pellet can thus be accounted for by phosphoprotein (alkali-labile P) and nucleic acids (hot TCA-extractable).

Acid-insoluble material from VC pellets was analysed by disc electrophoresis on SDS-polyacrylamide gels [9] after pre-incubation of the sample with SDS at 50° for 20 min. At the end of the run the gels were fixed in 10% TCA and proteins stained using Coomassie Brilliant Blue. After destaining, the gels were cut into sections and homogenised in ice-cold 10% TCA. After standing in ice for 30 min the gel residue and acid-insoluble materials were centrifuged down and the pellets washed twice with ice-cold 10% TCA to remove adhering inorganic phosphate. The residue was then incubated for 60 min at 37° with 1 M-NaOH to extract proteins and aliquots of the extracts were taken for estimation of protein and radioactivity (see sect. 2.6 and 2.7).

2.3.2. Ammonium sulphate fractionation (see [10])

After incubation, tissue was homogenised in four vol of 10 mM phosphate buffer, pH 6.8, containing 5 mM $MgCl_2$ (PMG buffer), with or without 0.24 M sucrose, and a high-speed supernatant prepared (10^5 *g* for 60 min). Protein was precipitated by addition of a saturated solution of ammonium sulphate and the fraction precipitating between 35% and 50% saturation was redissolved in homogenising buffer and incubated at 37° for 90 min in the presence of [^{3}H]colchicine (final conc., 2.5×10^{-6} M; specific radioactivity, 4.45×10^5 cpm/nmole). The labelled material was then analysed either by centrifugation for 18–20 hr at 100,000 *g* on a linear 5–20% sucrose gradient containing 10 mM phosphate buffer, pH 6.8, 5 mM $MgCl_2$, 0.1 M KCl, and 0.1 mM GTP, or by chromatography at 4° on a 20×1 cm column of Sephadex G-200 Superfine, equilibrated with PMG buffer. Fractions of 0.8 ml were collected during elution of the column with the same buffer.

2.4. *Purification of tubulin from pig brain*

Microtubular subunit protein was purified from pig brain by the batchwise procedure of Weisenberg et al. [10].

2.5. *Assay of bound phosphate*

Phosphoserine phosphorus of purified pig brain tubulin (see above) was determined as alkali-labile phosphate on 10 mg quantities of protein after precipitation with 10% TCA, extraction of the precipitate with lipid solvents, and alkaline hydrolysis as previously described [11]. Inorganic phosphate released by alkali was estimated by the method of Martin and Doty [12]. All determinations were carried out in duplicate.

In experiments where tissue was incubated with ^{32}P, the total acid-insoluble radioactivity of fractionated samples was measured after precipitation with 10% TCA in the presence of added carrier bovine serum albumin (0.5 mg/ml), and collection on glass fibre discs (Whatman GF/A). After washing with 50 ml 10% TCA the discs were transferred to glass scintillation vials and counted in 5 ml Bray's [13] scintillation mixture.

2.6. *Protein determination*

Protein was estimated by the method of Lowry et al. [14] using bovine serum albumin standards. With samples containing vincristine, protein was first precipitated with 10% TCA and washed with methanol to remove excess alkaloid (see [15]). In the estimation of protein in polyacrylamide gels, sections of a gel which had been run without sample were used as blanks, and similar sections to which albumin was added during the extraction procedure (see sect. 2.3) were used as standards.

2.7. *Counting techniques*

Radioactivity measurements were carried out in

5 ml Bray's solution [13] in a Packard 3375 spectrometer. Counting efficiences for ^{3}H and ^{32}P were 48 and 95%, respectively.

3. Results

3.1. *Electrophoresis in SDS-gels of VC-precipitable material*

The distribution of protein after SDS-polyacrylamide gel electrophoresis of a VC precipitate is shown in fig. 1. One major band was observed which, by comparison with markers of known molecular weight, was identified as "tubulin" monomer, (M.W. 58,000). Three minor bands of apparently lower molecular weight proteins were also observed. After electrophoresis of ^{32}P-labelled VC precipitates under the same conditions, and estimation of protein and acid insoluble radioactivity as described in sect. 2.3.1, two major peaks were observed (fig. 1), the faster of which coincided with the "tubulin" band. The slower-moving ^{32}P-labelled material did not appear to be associated with any protein, and indeed, was almost completely removed by prior extraction of the VC pellet with hot TCA (sect. 2.3.1); this treatment did not alter the protein distribution seen after staining. Furthermore, treatment of the precipitate with $CHCl_3$/MeOH (2/1, v/v) and ethanol/diethyl ether (1/1, v/v) affected neither protein nor radioactivity distribution. It can therefore be concluded that the peak of labelling associated with the "tubulin" band is not due to nucleic acids or lipids which might co-precipitate in the presence of vincristine.

It is apparent from fig. 1, however, that not all the bound ^{32}P that migrated more slowly than "tubulin" could be extracted by hot TCA. This material could

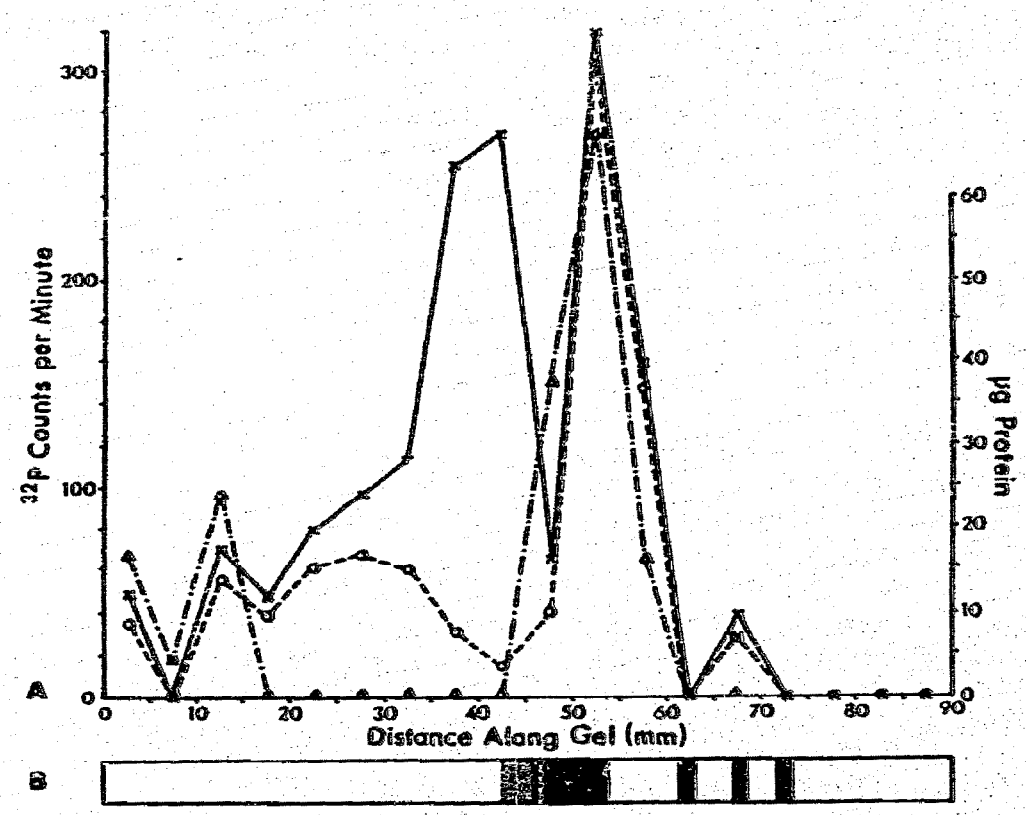

Fig. 1. A) Distribution of bound ^{32}P and protein in vincristine precipitates after electrophoresis in SDS-polyacrylamide gels. Bound ^{32}P and protein were determined as described in the text (sect. 2). (X—X—X) ^{32}P cpm; (o- - -o- - -o) ^{32}P cpm in hot TCA-treated material; (Δ- - -Δ- - -Δ) protein. B) Diagrammatic representation of SDS-polyacrylamide gels after staining proteins with Coomassie Brillant Blue. Stippled areas represent trailing of protein (due to overloading) behind main tubulin band migrating between 49–54 mm from origin.

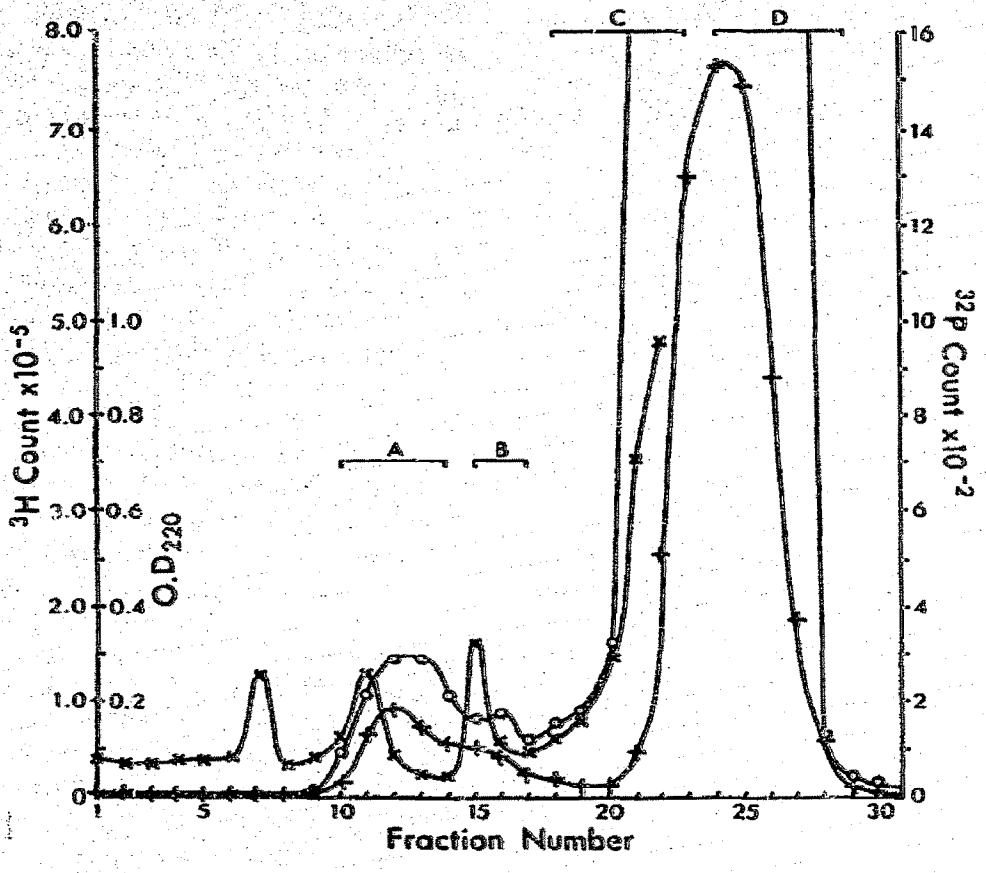

Fig. 2. Chromatography of crude tubulin preparation on Sephadex G-200 Superfine. Total ^{3}H and ^{32}P content was determined on aliquots of each fraction as described in the text (sect. 2), and the remaining samples were combined, as indicated in the figure (A–D), for determination of acid-insoluble ^{32}P (see table 1). (+——+——+) ^{3}H cpm × 10^{-5}; (o——o——o) ^{32}P cpm × 10^{-2}; (×——×——×) absorbance at 220 nm. The bulk of the free [^{3}H]colchicine was recovered in C and D.

not be detected after staining with Coomassie Blue and did not give any colour with the Lowry protein estimation. More sensitive methods will evidently be required in order to characterise it.

3.2. *Association of ^{32}P with tubulin dimers*

Electrophoresis under the conditions used above (sect. 3.2) dissociates microtubular subunit protein into its monomers. If one or both of these contain protein-bound phosphorus as suggested above (sect. 3.1), it should be possible to demonstrate the phosphorylation of the tubulin dimers from which they were derived. It was therefore decided to determine the extent of ^{32}P-binding to the colchicine-binding dimer by techniques which allow separation of the dimer using bound [^{3}H]colchicine as a marker, i.e. by gel filtration and sucrose gradient centrifugation (see [10]). However, Murray and Froscio [16] reported that, due to an interaction between phosphorylated tubulin and a soluble component of the high-speed supernatant material, purification beyond the second ammonium sulphate stage by DEAE-Sephadex (see [10]) could not be obtained. Ammonium sulphate fractions, labelled as described in sect. 2.3.2, were therefore used in our experiments.

3.2.1. Gel filtration

In fig. 2 are shown the distributions of protein (as measured by absorption at 220 nm) and the radioactivity due to both ^{3}H and ^{32}P. It is immediately evident that none of the three absorption peaks directly correspond to the main peaks of bound [^{3}H]col-

Table 1
Distribution of bound ^{32}P after chromatography of crude tubulin preparations on Sephadex G-200 Superfine.

Fraction	Total ^{32}P (cpm)	Acid-insoluble ^{32}P (cpm)
A	1100	336
B	450	170
C	3790	86
D	3090×10^3	0

Fractions from the Sephadex G-200 column were combined as shown in fig. 2. Total and acid-insoluble radioactivity were determined as described in the text.

chicine and ^{32}P. The main bound colchicine component (fraction A) in fact contains very little protein but is nevertheless associated with peaks of both total ^{32}P (fig. 2) and acid-insoluble ^{32}P (table 1). (Tubulin constitutes only a small proportion of the proteins separated by polyacrylamide gel electrophoresis of second ammonium sulphate precipitates (data not shown) and it is therefore not surprising that so little protein is associated with the bound colchicine.) The presence of a large amount of ^{32}P in the colchicine-

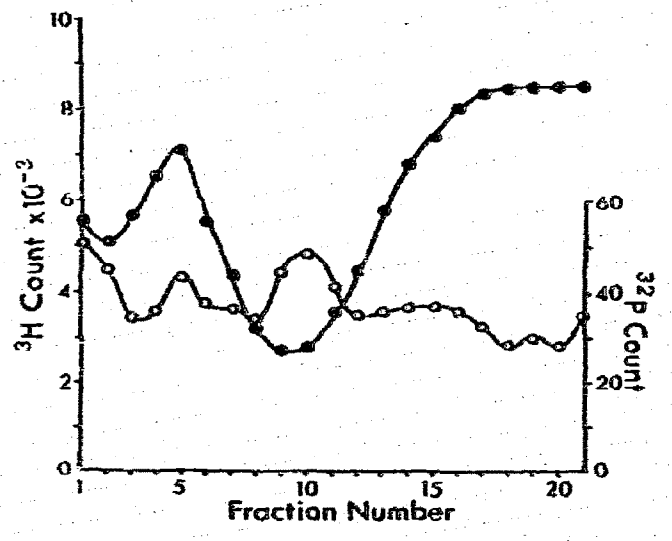

Fig. 3. Sucrose gradient centrifugation of crude tubulin preparation. After centrifugation as described in the text (sect. 2), fractions of 5 drops each were collected and 0.1 ml aliquots taken for determination of total tritium cpm, (●—●—●), the remainder of each fraction was used to determine acid-insoluble ^{32}P cpm (○—○—○). Fraction 5 contains the peak of protein-bound colchicine, while ^3H cpm beyond fraction 10 represent free colchicine (see [10]).

binding fraction suggests the likelihood that the tubulin dimer is phosphorylated. To obtain further information about the extent of phosphorylation of the colchicine-binding dimer, an alternative approach using sucrose density gradients, was employed.

3.2.2. Sucrose gradient centrifugation

The distributions of [^{3}H]colchicine and acid-insoluble ^{32}P after sucrose density gradient centrifugation of a second ammonium sulphate pellet (see sect. 2.3.2) are shown in fig. 3; a clear separation of bound from free colchicine was obtained. A significant amount of bound ^{32}P was associated with fractions enriched in colchicine-binding dimer, although it is apparent that acid-insoluble ^{32}P was distributed throughout the gradient. In this and other experiments, unexpectedly high counts due to ^{32}P and ^3H were found at the bottom of the centrifuge tube (fig. 3, fraction 1) (see Discussion).

3.3. *Phosphate content of tubulin*

Purified tubulin from pig brain contained 0.33 and 0.40 moles alkali-labile phosphate per mole of dimer in two separate preparations. Alkaline hydrolysis under these conditions has been shown, even in whole tissue extracts, to release P_i mainly from phosphoserine and phosphothreonine [17]. Moreover, acid hydrolysis of purified tubulin after incubation *in vitro* with ATP (γ-^{32}P) has shown phosphoserine to be the only labelled amino acid recovered (see [6]; Lagnado and and Weller, unpublished observations). It is therefore concluded that the phosphorus released from purified tubulin by alkaline hydrolysis is most probably derived from phosphoserine, and that the levels reported are those of phosphoserine phosphorus.

4. Discussion

Microtubular protein in crude tissue extracts has been frequently identified by its mobility in SDS-polyacrylamide gels with reference to the 58,000 MW tubulin monomer isolated on the basis of its affinity for colchicine, (see [3]). In the present work it was shown that after addition of vincristine to soluble extracts of incubated tissue, which precipitates virtually all of the colchicine-binding protein (see sect. 2.3.1), subsequent electrophoresis of the alkaloid-induced

aggregate under dissociating conditions separates a main protein band, co-migrating with purified tubulin monomer, which contained most of the protein-bound ^{32}P (fig. 1). Moreover, it was calculated that about 20% of the total ^{32}P-labelled protein-bound phosphorus in the supernatant obtained from incubated slices was recoverable in the VC precipitate, which accounted for only ca. 10% of the total soluble protein. It is therefore evident that the protein characterised as tubulin exhibits a significant turnover of protein-bound phosphate in an intact cell preparation studied under conditions where ATP and creatine phosphate levels are maintained (see [7]).

Further evidence that phosphorylation of tubulins occurs in intact cells was obtained in experiments in which protein-bound ^{32}P was estimated in microtubular subunit protein characterised by its colchicine-binding activity after separation by gel filtration and sucrose gradient centrifugation (figs. 2 and 3; table 1). It was noted that a significant amount of bound ^{32}P was also associated with material which sedimented more rapidly than the main colchicine-binding component on sucrose gradients (fig. 3). The interpretation of this finding awaits further characterisation of the fast sedimenting material, but it may be relevant that ^{32}P was recently detected in association with rapidly sedimenting material present in purified preparations of tubulin which had been labelled *in vitro* with [γ-^{32}]-ATP [20].

A further observation requiring clarification concerns the low levels of phosphoserine phosphorus (about 0.36 moles P/mole tubulin dimer) detected in isolated tubulin preparations from pig brain (see sect. 3.3).

One might reasonably expect that, even if only one of the two forms of tubulin monomers present in the colchicine-binding dimer were phosphorylated, a value closer to one mole P/mole of dimer would be obtained. One possible explanation which has not been completely excluded is that the phosphorylated component is a contaminant which co-purifies with tubulin during its isolation. This seems unlikely since virtually all of the bound ^{32}P detected after electrophoresis in SDS-gels coincided with tubulin monomer separated after labelling the purified protein with [γ-^{32}P]ATP [3, 6] *in vitro* or with [^{32}P]orthophosphate in a cell-containing preparation (sect. 3.1, fig. 1). A more likely explanation is that the tubulin preparation contains a mixture of phosphorylated and non-phosphorylated dimers, which were not separated by the purification methods employed. It has in fact been shown that phosphorylation of tubulin appears to have marked effect on its behaviour and recovery during the isolation procedure [16] and further work will be required to interpret the quantitative aspects of tubulin phosphorylation reported here.

An obvious question relating to the present results, which we are now investigating, concerns whether the phosphorylation of tubulins in intact tissue occurs in the polymerised subunits or in the 'free' pool. Such information may help to show whether phosphorylation plays a part in controlling the assembly of microtubules during the establishment of functional connections in nerve tissue.

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