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Phosphorylation of Pig Brain Microtubule Proteins

GENERAL PROPERTIES AND PARTIAL CHARACTERIZATION OF ENDOGENOUS SUBSTRATE AND CYCLIC AMP-DEPENDENT PROTEIN KINASE

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1. A simple purification procedure for microtubule proteins is described, which involves a single assembly step *in vitro* in the absence of glycerol, followed by centrifugation through sucrose. 2. The preparation contains 80% tubulin (mol.wt. 54000), 15–20% of a 280000-mol.wt. protein and several other minor components of intermediate molecular weight after polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate and 2-mercaptoethanol. 3. In the presence of [γ-^{32}P]ATP, [^{32}P]phosphate was incorporated into the 280000-mol.wt. component reaching half-maximal incorporation at 1–2min, but no phosphorylation of tubulin was detected. Cyclic AMP (K_m 0.8μM) increased both the initial rate and the extent of incorporation of [^{32}P]phosphate into this component. 4. About half of the endogenous protein kinase activity did not require cyclic AMP and was not inhibited by a heat-stable inhibitor protein from muscle. The remainder of the activity was cyclic AMP-dependent and sensitive to the inhibitor protein. A regulatory subunit was not dissociable from microtubules assembled *in vitro* in the presence of saturating concentrations of cyclic AMP. 5. The endogenous substrate and the endogenous protein kinase activity could be partially resolved by chromatography on phosphocellulose. 6. The data show that cyclic AMP can modulate the activity of an endogenous protein kinase(s) with unusual properties and which phosphorylates a prominent microtubule-associated protein.

Cytoplasmic microtubules are dynamic structures that can respond to specific changes in cellular requirements (Roisen *et al.*, 1972; Willingham & Pastan, 1975; Pipeleers *et al.*, 1976; Sheterline *et al.*, 1977). Studies with antimitotic drugs have implicated these structures in many intracellular transport phenomena (Inoue & Sato, 1967; Murphy & Tilney, 1974; Sheterline *et al.*, 1975; Yahara & Edelman, 1975), in which it is believed that modulation of microtubule assembly and interaction with other cell structures may be necessary. Tubulin assembly *in vitro* is sensitive to Ca^{2+} ions (Weisenberg, 1972; Olmsted & Borisy, 1975), thiol oxidation (Mellon & Rebhun, 1976) and the presence of accessory proteins (Murphy & Borisy, 1975; Weingarten *et al.*, 1975). An enzyme is also present in extracts from several tissues which specifically incorporates tyrosine on to the carboxyl terminal of the α-subunit of tubulin, although without any apparent effect on assembly (Raybin & Flavin, 1977). However, none of these mechanisms have yet been shown to operate *in vivo*.

Since the demonstration of tubulin assembly from tissue extracts *in vitro* (Weisenberg, 1972) and the wide usage of an isolation method based on this procedure (Shelanski *et al.*, 1973), it has become evident that other proteins (Borisy *et al.*, 1975), including protein kinase activity (Sloboda *et al.*, 1975), are associated with microtubules assembled *in vitro* and co-purify with tubulin through several cycles of assembly/disassembly. The presence of an integral cyclic AMP-sensitive microtubule-phosphorylating system could also provide a control mechanism for an as yet undefined function not related to assembly (Rappaport *et al.*, 1976; Sheterline, 1976).

Endogenous phosphorylation of at least two proteins which co-purify with tubulin (Sloboda *et al.*, 1975; Sheterline, 1976; Rappaport *et al.*, 1976) and of tubulin itself (Goodman *et al.*, 1970; Lagnado *et al.*, 1975; Castle & Crawford, 1976) has been demonstrated *in vitro*, and in both microtubules purified by assembly from pituitary tissue (Sheterline & Schofield, 1975) and by affinity chromatography from brain (Sandoval & Cuatrecasas, 1976) phosphoprotein phosphatase activity was present. The nature of the endogenous substrate *in vitro* has not been clearly established, and its role, if any, *in vivo* is unknown. The present paper describes a simple preparation procedure for microtubules from pig brain and

the partial characterization of the endogenous protein kinase(s) and substrate.

Materials and Methods

Chemicals

[γ-^{32}P]ATP was purchased from The Radiochemical Centre, Amersham, Bucks., U.K., in 50% (v/v) ethanol. The ethanol was removed under vacuum and the [γ-^{32}P]ATP stored as a 1mM solution in 0.25M-Pipes,* pH6.8, at −30°C and at an initial specific radioactivity of 250mCi/mmol. Butyl-PBD was obtained from Birchover Instruments, Letchworth, Herts., U.K., and incorporated into a scintillation 'cocktail' containing 6g of butyl-PBD, 80g of naphthalene, 600ml of toluene and 400ml of 2-methoxyethanol. GTP and phosphorylase *b* were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Phosphocellulose type P-11 was obtained from Whatman Lab Sales, Maidstone, Kent, U.K., and all other chemicals and materials for electrophoresis were obtained from BDH Chemicals, Poole, Dorset, U.K.

Preparation of microtubule proteins

Pig brain was homogenized by using a rotary tissue mincer (International Laboratory apparatus X-1020) into an equal volume of 50mM-Pipes/5mM-2-mercaptoethanol/0.1mM-$MgCl_2$/0.1mM-EGTA adjusted to pH6.8 with NaOH at 23°C. The homogenate was centrifuged for 60min at 25000$g_{av.}$ in a centrifuge maintained at 2°C, and the supernatant, which contained most of the tubulin, was retained. To initiate assembly of microtubule proteins, the supernatant was made 1mM with respect to GTP and incubated at 37°C for 30min. The viscous supernatant was then layered on an equal volume of 20% (w/v) sucrose in homogenizing buffer maintained at 37°C, and centrifuged at this temperature for 30min at 40000$g_{av.}$ The large translucent pellet was gently washed with water and homogenized with a Potter Teflon/glass homogenizer in 50mM-Pipes/5mM-2-mercaptoethanol/0.1mM-$MgCl_2$, pH6.8, to give a protein concentration of about 5mg/ml of buffer. The microtubules in the preparation were left on ice for either 60min or overnight to depolymerize, after which time cold-insoluble structures were removed by centrifugation for 30min at 40000$g_{av.}$ and 2°C. The supernatant could be stored for 2–3 days on ice. Stages during the purification procedure were monitored by electron microscopy.

Protein destined for chromatography on phosphocellulose was homogenized in 50mM-sodium phosphate/5mM-2-mercaptoethanol/0.1mM-$MgCl_2$, pH6.5, and dialysed against 100vol. of the same buffer overnight at 4°C. Insoluble structures were removed by centrifugation as detailed above.

* Abbreviations: butyl-PBD, 5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole; Pipes, 1,4-piperazinediethanesulphonic acid; SDS, sodium dodecyl sulphate; ATPase, adenosine triphosphatase.

Measurement of microtubule protein kinase activity

Samples of the microtubule preparation were incubated at a final concentration of 1mg/ml in 50mM-Pipes/2.5mM-$MgCl_2$/0.1mM-[γ-^{32}P]ATP/50μM-3-isobutyl-1-methylxanthine, pH6.8, at 37°C for the times indicated in the legends to the Figures and with additions specified therein. The addition of methylxanthine, which did not affect protein kinase activity, prevented hydrolysis of added cyclic AMP by endogenous phosphodiesterase activity (Beavo *et al.*, 1970). Trichloroacetic acid-insoluble [^{32}P]phosphate was measured essentially by the method of Corbin & Reimann (1974). Samples (15μl) of the reaction mixture were layered on rectangles (2.5cm×1cm) of Whatman 3MM paper and the rectangles washed in an expanded stainless-steel basket suspended in a beaker and stirred continuously by magnetic stirrer. One 30min wash in ice-cold 10% (w/v) trichloroacetic acid and one 30min wash in ice-cold 5% trichloroacetic acid were used, with approx. 15ml of acid per rectangle. The rectangles were then washed in acetone and added to 5ml of butyl-PBD scintillator for determination of radioactivity. Since the specific radioactivity of [γ^{32}P]ATP decreased throughout a batch of experiments, an internal standard was included for measurement of specific radioactivity in each experiment.

Gel electrophoresis

Determination of subunit molecular weight. Molecular-weight determinations were made by electrophoresis in gels containing 4.0% (w/v) acrylamide and 0.11% (w/v) *NN'*-methylenebisacrylamide and the phosphate-buffer system of Weber & Osborn (1969), containing 0.1% (w/v) SDS. Standard proteins myosin, phosphorylase, bovine serum albumin, tubulin and actin were assumed to have subunit mol.wts. of 200000, 90000, 68000, 54000 and 43000 respectively.

Separation of microtubule proteins. All other electrophoresis experiments were performed in the buffer system, of Laemmli (1970) in the presence of 0.1% (w/v) SDS with 6% (w/v) acrylamide and 0.16% (w/v) *NN'*-methylenebisacrylamide gels. This buffer system gives improved resolution and provides conditions under which the two tubulin monomers, designated α-and β-tubulin, exhibit different mobilities (Bryan, 1974). Relative protein concentrations were estimated by planimetry of densitometric scans of Coomassie Blue-stained gels.

Distribution of [^{32}P]phosphate in polyacrylamide gels. Gels were frozen on solid CO_2 to a toffee-like consistency and sliced into sections by using razor blades set 2mm apart. Sections were dissolved by the

addition of 0.5ml of 30% (w/v) H_2O_2 and incubated for 3–4h at 80°C. The residue was dissolved in 0.1ml of 0.1M-HCl and radioactivity measured in a liquid-scintillation spectrometer, after the addition of 8ml of water, by Cerenkov radiation at an efficiency of 40–50%. Some gels were run by using *NN'*-diallyltartardiamide instead of *NN'*-methylenebisacrylamide, at the same molar concentration, to take advantage of the rapid solubilization of the former polymer by periodate at room temperature (Anker, 1970). However, the gels are 'sticky' and difficult to handle and at the same monomer concentration permit higher mobilities of protein–SDS complexes.

Preparation of ^{32}P-labelled phosphorylase a. Phosphorylase *a* was prepared from phosphorylase *b* in the presence of phosphorylase kinase (Walsh *et al.*, 1971). Phosphorylase kinase was activated by incubation for 20min at 30°C in the presence of 10μM-cyclic AMP, 10μM-$CaCl_2$ and 0.1mM-ATP. Incubations containing 4mg of phosphorylase *b*/ml and 20μg of phosphorylase kinase/ml were performed in 25mM-2-glycerophosphate / 25mM-Pipes / 15mM-2-mercaptoethanol/2mM-$MgCl_2$ at pH7.0. [γ-^{32}P]ATP was added to give a twofold molar excess over phosphorylase *b*. The reaction was essentially completed by 40min at 30°C. Typical preparations were 50–60% phosphorylated at a specific radioactivity of 10^3c.p.m./μg of phosphorylase. Samples were dialysed overnight at 4°C against the same buffer before use.

Chromatography on phosphocellulose

Phosphocellulose was precycled by washing initially in 50% (v/v) ethanol (as a 20% gel/ethanol slurry), then with 15vol. of 0.5M-NaOH followed by 100vol. of water. The alkaline wash was followed by a similar wash with 0.5M-HCl. The phosphocellulose was equilibrated with 50mM-sodium phosphate/5mM-2-mercaptoethanol / 0.1mM-$MgCl_2$, pH6.8, before use. The gel was packed into a column (2cm×15cm) (45ml) and eluted at a constant flow rate of 0.2ml/min; 2.5ml fractions were collected.

After dialysis microtubule proteins were adjusted to 4mg/ml of buffer and loaded on the column. Most of the protein was eluted with column buffer and the remainder by a linear 300ml 0–0.75M-NaCl gradient in the same buffer. Fractions obtained were dialysed overnight against 200vol. of column buffer to remove most of the NaCl before analysis. All steps for chromatography and preparation were performed at 4°C.

Assay of column fractions. (*a*) Cl^- concentration in column eluates was estimated titrimetrically by using the Sigma chloride-assay reagents (Schales & Schales, 1941).

(*b*) ATPase activity was measured by separating [^{32}P]phosphate from [γ-^{32}P]ATP in the presence of 10% (w/v) Norit A charcoal in 5% (v/v) $HClO_4$ with carrier 1M-NaH_2PO_4. [γ-^{32}P]ATP adsorbed to the charcoal (Tsuboi & Price, 1959) was removed by centrifugation in a Beckman Microfuge (2min at 10000$g_{max.}$) and samples of the supernatant were assayed for radioactivity with 8ml of water by Cerenkov radiation.

(*c*) To avoid concentrating column fractions to provide sufficient incorporation to detect by the assay procedure of Corbin & Reimann (1974), endogenous phosphorylation in column fractions was determined by incubation of 250μl samples for 20min at 37°C containing 120μl of desalted column fraction, 100μM-[γ-^{32}P]ATP, 2.5mM-$MgCl_2$, with or without 10μM-cyclic AMP, in column buffer. The reaction was stopped by addition of 250μl of 20% (w/v) trichloroacetic acid and the insoluble material filtered through Whatman GF/A glass-fibre discs. The discs were washed with 8ml of ice-cold 5% (w/v) trichloroacetic acid and the radioactivity on the filters was measured in the presence of 5ml of butyl-PBD scintillator.

(*d*) Protein concentration was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Electron microscopy

Supernatant microtubules were layered on 300-mesh copper grids coated with cellodion and negatively stained with 1% (w/v) uranyl acetate. Pellets were fixed in 2% (v/v) glutaraldehyde/8% (w/v) tannic acid / 100mM-sodium phosphate (pH6.5) (Tilney *et al.*, 1973) at 37°C for 4h. Pellets were post-fixed in buffered OsO_4, dehydrated with graded alcohols and propylene oxide, embedded in Epon and sectioned.

Results and Discussion

Isolation of pig brain microtubules

Borisy *et al.* (1975) have reviewed various requirements for the isolation of microtubule proteins by assembly *in vitro*. Modifications to the original Shelanski *et al.* (1973) procedure included omission of glycerol from the assembly medium, which increased the variable stoicheiometry of tubulin-associated high-molecular-weight protein(s) from 4–12% (w/w) (Shelanski *et al.*, 1973; Gaskin *et al.*, 1974; Sloboda *et al.*, 1975) to 15–20% (w/w) (Borisy *et al.*, 1974). This latter stoicheiometry is supported by the morphological studies of Amos (1977).

In the procedure described here, replacement of the second polymerization cycle of Borisy *et al.* (1974) by centrifugation through sucrose, and inclusion of 2-mercaptoethanol in the assembly buffer (Mellon & Rebhun, 1976), results in shorter isolation times, and higher yields (1mg/g of brain tissue) of an otherwise similar preparation. Observation by electron microscopy of negatively stained material from

the assembly step before centrifugation, and of thin sections of the pellet, show only structures morphologically similar to microtubules. The nature of the high-molecular-weight microtubule proteins is difficult to establish, since estimates of molecular weights in this range by polyacrylamide-gel electrophoresis in the presence of SDS are unreliable, and there is some evidence for proteolytic degradation of these proteins (Sloboda *et al.*, 1976). However, the short isolation time for this procedure produces a single major band, which constitutes 15–20% of the total protein present, by electrophoresis in the gel system of Laemmli (1970) and which migrates in 4% (w/v) acrylamide gels containing 0.1% SDS at a position corresponding to a mol.wt. of 280000 (results not shown).

Endogenous phosphorylation by microtubule preparations

[^{32}P]Phosphate was rapidly incorporated into trichloroacetic acid-insoluble material from [γ-^{32}P]-ATP by the microtubule preparation, and both the initial rate and the final extent of incorporation were increased by cyclic AMP (Fig. 1). The apparent K_m for the enhancement by cyclic AMP of the rate of [^{32}P]phosphate incorporation by the preparation (0.8 μM) is close to values reported for both bovine brain (Goodman *et al.*, 1970) and chick brain (Sloboda *et al.*, 1975) microtubule preparations. Addition of excess of unlabelled ATP to the incubation medium resulted in a small decrease in the amount of trichloracetic acid-insoluble [^{32}P]phosphate detected, followed by a slow ($t_{\frac{1}{2}} > 60$ min) linear decrease. The whole preparation contains low ATPase activity (<10 nmol/min per mg), and enzymic studies have shown that the trichloroacetic acid-insoluble [^{32}P]phosphate is over 85% trypsin-sensitive and about 13% phospholipase C-sensitive, indicating that this small decrease on addition of unlabelled ATP could be due to turnover of phospholipid (Kirazov & Lagnado, 1976). The slow linear decrease in [^{32}P]phosphate is presumably due to the presence of phosphoprotein phosphatase, although no such activity could be detected with exogenous ^{32}P-labelled phosphorylase *a* as substrate (Sheterline & Schofield, 1975). Phosphoprotein phosphatase activity has been found in both pituitary microtubule preparations (Sheterline & Schofield, 1975) and in tubulin prepared from brain by affinity chromatography on colchicine linked to Sepharose (Sandoval & Cuatrecasas, 1976). In the latter preparation the phosphatase activity was enhanced in the presence of 1 nM–0.1 μM-cyclic GMP; however, addition of 8 nM-cyclic GMP to this system neither decreased incorporation nor increased the rate of loss of trichloroacetic acid-insoluble [^{32}P]phosphate after addition of unlabelled ATP (Fig. 1).

Analysis of [^{32}P]phosphate incorporation into

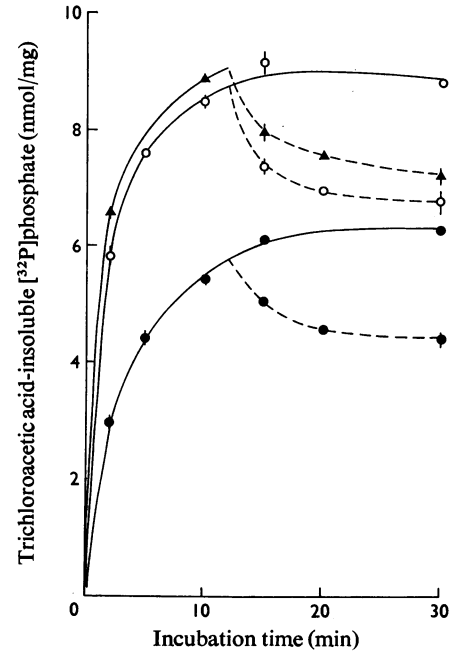

Fig. 1. *Time course for endogenous incorporation of [^{32}P]phosphate from [γ-^{32}P]ATP into microtubule trichloroacetic acid-insoluble material*

Microtubule proteins were endogenously phosphorylated for the times indicated as described in the Materials and Methods section. ●, Control; ○, 10 μM-cyclic AMP; ▲, 10 μM-cyclic AMP with 8 nM-cyclic GMP. ----, Addition of excess of unlabelled ATP (to 2 mM) added at 12 min. Data are expressed as means ± S.E.M. for a total of four determinations on two different preparations.

microtubule proteins separated by polyacrylamide-gel electrophoresis in the presence of SDS showed that most of the [^{32}P]phosphate co-migrated with the major high-molecular-weight protein (Fig. 2), of mol.wt. 280000 by the gel system described. Small amounts of [^{32}P]phosphate migrated with apparent mol.wts. approx. 70000, but almost none migrated with either of the tubulin subunits separated by using the Laemmli (1970) buffer system. It is possible that the sensitivity of these high-molecular-weight proteins to proteolysis (Sloboda *et al.*, 1976) results in a range of phosphorylated fragments of lower molecular weight, since no [^{32}P]phosphate migrated behind the major band.

Properties of the endogenous protein kinase(s)

Traugh *et al.* (1974) have laid down criteria for the classification of protein kinases based on their sensitivity to both cyclic AMP and the heat-stable inhibitor protein (Ashby & Walsh, 1972). About half of the

protein kinase activity in this preparation was expressed in the absence of cyclic AMP (Fig. 2) and was unaffected by the heat-stable inhibitor protein (Fig. 3) which binds to the catalytic subunit of cyclic AMP-dependent protein kinases (Ashby & Walsh, 1972). Thus this activity could be classified in group III by using the system of Traugh *et al.* (1974). The cyclic AMP-dependent activity was, in contrast, completely blocked by the heat-stable inhibitor protein (Fig. 3) and thus appears to belong with other cyclic AMP-dependent protein kinase holoenzymes in group I. Experiments were designed to dissociate the postulated regulatory subunit from the microtubule kinase in the presence of cyclic AMP (Brostrom *et al.*, 1970). Three experimental approaches were used: (i) microtubules were assembled from pig brain extracts in the presence of cyclic AMP, and collected after centrifugation through sucrose; (ii) as in experiment (i), but with the addition of an intermediate layer of sucrose containing cyclic AMP through which the microtubules were centrifuged; and (iii) the experiment shown in Fig. 4, in which purified microtubule proteins were assembled for a second time, but addition of a saturating concentration (20 μM) of cyclic AMP was made 2 min before centrifugation through sucrose. In each case the sedimented microtubule preparation retained its full cyclic AMP-dependence. Assay of the assembly medium for cyclic AMP (Brown *et al.*, 1971) after centrifugation showed that at least 60% of the added nucleotide was still present, which excludes hydrolysis of cyclic AMP by the phosphodiesterase activity present. The data may indicate the presence of specific microtubule protein kinase. These data differ somewhat from those published by Rappaport *et al.* (1976) for rat brain microtubules purified by assembly in the presence of glycerol (Shelanski *et al.*, 1973). The properties of the

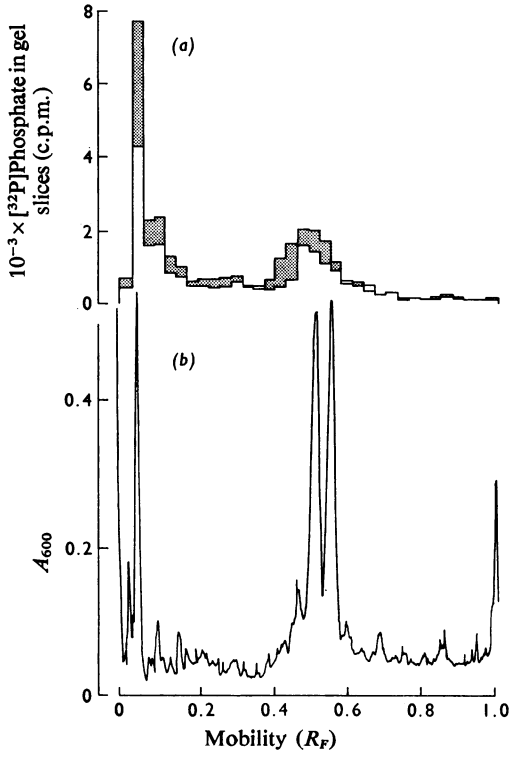

Fig. 2. *Distribution of* [^{32}P]*phosphate in microtubule components separated on Laemmli* (1970) *gels*
(*a*) Samples of microtubule proteins, prepared as described in the Materials and Methods section, were endogenously phosphorylated with [γ-^{32}P]ATP (540 c.p.m./pmol) for 12 min in the absence (open columns) and presence (stippled columns) of 10 μM-cyclic AMP. After incubation, rapidly turning over trichloroacetic acid-insoluble [^{32}P]phosphate was displaced by incubation for a further 10 min in the presence of excess (2 mM) of unlabelled ATP (Fig. 1). (*b*) Samples (15 μg) were subjected to electrophoresis in the presence of SDS on 6% (w/v) acrylamide gels by using the buffer system of Laemmli (1970). Gels were scanned at A_{600} (——) by using a Gilford linear transport; mobility is expressed relative to a Bromophenol Blue marker ($R_F = 1.0$).

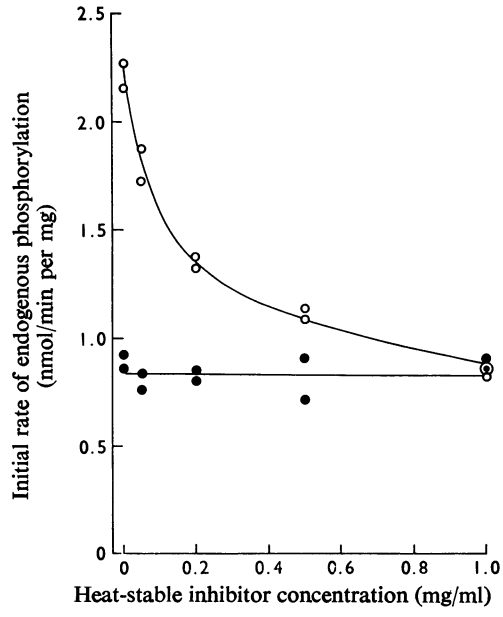

Fig. 3. *Effect of heat-stable inhibitor protein on endogenous phosphorylation of microtubule proteins*
Microtubule proteins were incubated in the absence (●) and presence (○) of 10 μM-cyclic AMP as described in the Materials and Methods section for the measurement of endogenous phosphorylation. Incubation mixtures also contained concentrations of heat-stable inhibitor protein as detailed on the Figure.

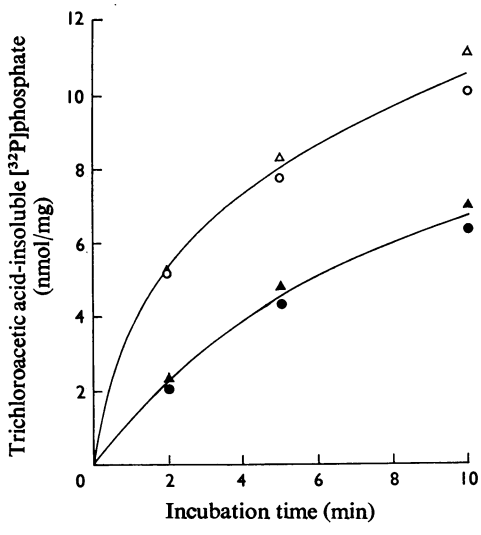

Fig. 4. *Effect of attempted dissociation of regulatory subunit on cyclic AMP-dependence of microtubule preparation*
Microtubule proteins were prepared as described in the Materials and Methods section, then submitted to a second assembly cycle identical with the first in the presence (▲, △) or absence (●, ○) of 20 μM-cyclic AMP to dissociate a postulated protein kinase regulatory subunit. The microtubules thus assembled were then centrifuged through sucrose. The cyclic AMP-dependency for endogenous phosphorylation in these two preparations was then assayed over the time course detailed in the absence (●, ▲) and presence (○, △) of 10 μM-cyclic AMP. Results are the means of duplicate determinations at each time point.

microtubule-associated protein kinase activity described therein conform to those expected for a class I holoenzyme (Traugh *et al.*, 1974), and phosphorylation of at least two high-molecular-weight components occurs. The plateau value of [^{32}P]phosphate incorporation into the preparation in the presence of [γ-^{32}P]ATP was also less than 10% of that reported here. It appears that the method used for assembly *in vitro* has significant effects on the properties of the microtubules prepared. Unfortunately, no direct comparison between the properties of microtubules assembled *in vitro* and microtubules *in situ* is yet possible.

Separation of endogenous substrate and protein kinase activity

The accessory proteins of microtubules can be separated from tubulin by chromatography on phosphocellulose (Weingarten *et al.*, 1975) and by using an extended salt gradient, they can be partially resolved from each other (Fig. 5). Since most of the [^{32}P]phosphate is incorporated into the 280000-mol.wt. component by the endogenous kinase, samples (<1%) of the material for chromatography were labelled in the presence of [γ-^{32}P]ATP to act as a marker for the high-molecular-weight protein. The peak of radioactivity was consistently eluted at 0.26 M-NaCl and roughly corresponded to one of the protein peaks. The coincidence of the 280000-mol.wt. component with the [^{32}P]phosphate peak was confirmed by using SDS/polyacrylamide-gel electrophoresis. Shigekawa & Olsen (1975) have shown by gel

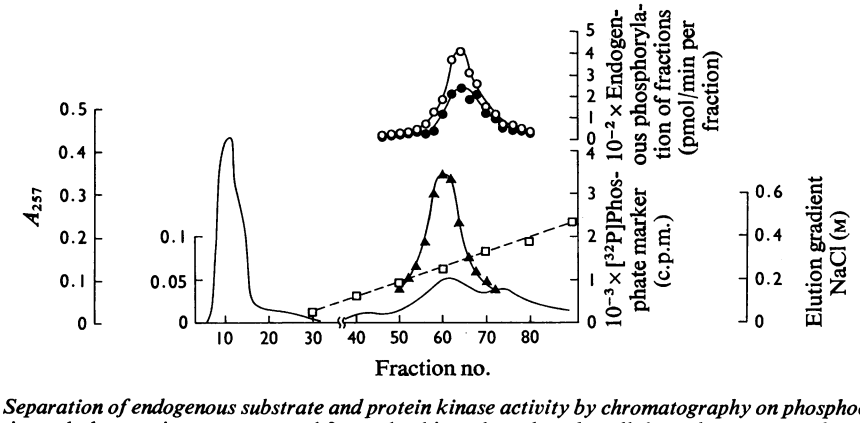

Fig. 5. *Separation of endogenous substrate and protein kinase activity by chromatography on phosphocellulose*
Samples of microtubule protein were prepared for and subjected to phosphocellulose chromatography as described in the Materials and Methods section. The elution position of the endogenous substrate was determined by endogenously phosphorylating a small portion (<1%) of the added protein in the presence of [γ-^{32}P]ATP. [^{32}P]Phosphate was measured directly in column eluates by Čerenkov radiation (▲). Endogenous phosphorylation by dialysed fractions was measured in the absence (●) and presence (○) of 10 μM-cyclic AMP as described in the Materials and Methods section and is shown in the upper part of the Figure. A_{257} for fractions 37 onwards is plotted on an expanded scale to show more clearly the elution characteristics of the relatively small amounts of protein eluted by the NaCl gradient (□).

filtration of microtubules prepared from mouse brain, that endogenous protein kinase activity was eluted at a position corresponding to a mol.wt. of 280000. The apparent peak of endogenous protein kinase activity (no exogenous substrate added) did not, however, co-migrate with the substrate, but was eluted just behind, at 0.28M-NaCl. Presumably this peak of cyclic AMP-dependent protein kinase activity reflects only the overlap between the kinase and substrate peaks. This procedure may thus form a basis for the isolation of components from a microtubule-phosphorylating system.

The physiological role for phosphorylation of this 280000-mol.wt. component is unknown, but it does not appear to alter either the extent (Sheterline, 1976) or the time course (Rappaport *et al.*, 1976) of tubulin assembly *in vitro*. The high molecular weight of this polypeptide allows some comparison with the ATPase protein dynein, associated with flagellar doublet microtubules, but the ATPase activity associated with other microtubule preparations assembled *in vitro*, is not associated with this protein (Burns & Pollard, 1974; Gaskin *et al.*, 1974), and in the present study no ATPase activity was detected in fractions eluted from phosphocellulose by the salt gradient.

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