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Rat Brain Microtubule Protein: Purification and Determination of Covalently Bound Phosphate and Carbohydrate

(subunits/phosphoserine/tissue culture/colchicine binding)

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ABSTRACT **Microtubule protein purified from rat brain by published procedures contains nucleic acid and carbohydrate contaminants. Microtubule protein isolated from the soluble fraction of rat brain homogenates by a new procedure contains no covalently bound amino sugars and no more than 0.2% neutral sugar, but does contain covalently bound phosphate. Total phosphate analysis of rat brain microtubule protein indicates the presence of 0.8 mol of phosphate per mol of protein dimer (110,000 g). The presence of covalently bound phosphate has been confirmed by preparation of ^{32}P-labeled microtubule protein from rat brain slices maintained in tissue culture in the presence of [^{32}P]orthophosphate. Furthermore, phosphorylation is specific to the more negatively charged microtubule protein subunit. ^{32}P-Labeled serine phosphate has been identified after partial acid hydrolysis of labeled microtubule protein.**

For the study of the control of microtubule assembly it is essential to know whether covalent modification of the protein subunits occurs. Microtubule protein can serve as a substrate for various protein kinases (1, 2), but the state of phosphorylation of soluble microtubule protein purified from brain has not been determined. Covalent modification might also occur by attachment of carbohydrate residues. Falxa and Gill (3) and Goodman *et al.* (2) have reported that microtubule protein from bovine brain is a glycoprotein, and Feit *et al.* (4) reported the presence of hexosamines in microtubule protein from mouse brain. In this laboratory, microtubule protein isolated from the soluble fraction of rat brain homogenates by the method of Weisenberg, Borisy, and Taylor (5) was found to contain phosphate, neutral sugars, and amino sugars. Further purification done here showed that rat brain microtubule protein prepared in this manner is contaminated by nucleic acid and carbohydrate that give rise to erroneous estimates of bound phosphate and carbohydrate.

To minimize the amount of contaminant present, purification was done in pyrophosphate buffers, in a modification of the published procedure. Microtubule protein prepared in this manner did not contain amino sugars, but low amounts of phosphate were still detected. The low amounts of phosphate could be due to protein-bound phosphate or to a remaining low level (0.2–0.3% on a weight basis) of nucleic-acid contamination. In order to distinguish between these possibilities, rat brain slices were grown in tissue culture in the presence of [^{32}P]orthophosphate and a tritiated marker amino acid. Microtubule protein was isolated from these slices and assayed for the presence of covalently bound ^{32}P by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS) or SDS and urea.

Abbreviations: SDS, sodium dodecyl sulfate; PPMg, 0.05 M sodium pyrophosphate–2.5 mM $MgCl_2$ (pH 7.0).

MATERIALS AND METHODS

Purification of Rat Brain Microtubule Protein. Microtubule protein was prepared by a modification of the method of Weisenberg *et al.* (5). Pyrophosphate buffers were used throughout to minimize the interaction of microtubule protein with nucleic acids. Brains from adult rats (Charles River Breeding Laboratories, Inc.) were minced and homogenized at 0–4° in 0.05 M sodium pyrophosphate–2.5 mM $MgCl_2$–0.24 M sucrose–0.1 mM GTP, pH 6.5 (at room temperature). The soluble protein (separated at 16,000 × *g*, 30 min) was fractionated with neutralized (pH 6.5 after 20:1 dilution), saturated ammonium sulfate. The fraction precipitating between 35 and 50% saturation was taken up in 0.05 M sodium pyrophosphate–2.5 mM $MgCl_2$ (pH 7.0) (PPMg) and run onto a DEAE-cellulose column (Whatman DE52, column volume 3 ml/g wet weight) equilibrated with PPMg. The bulk of the protein was eluted with PPMg plus 0.10 M NaCl, and microtubule protein was eluted with PPMg plus 0.26 M NaCl. DEAE-purified microtubule protein was further purified on Bio-Gel A-1.5 m (Bio Rad) in PPMg–0.1 M KCl–0.1 mM dithiothreitol (pH 7.0).

Preparation of Labeled Microtubule Protein. Newborn rats were decapitated, and the brain and brain stem (0.2–0.3 g wet weight) were removed and placed in chilled L-15 medium (6). The tissue was cleaned of meninges and diced into 1-mm cubes. The chunks from two brains were washed twice with incubation medium (L-15 deficient in orthophosphate and tyrosine, 5% adult rat serum, 0.3% bovine serum albumin, sodium bicarbonate*) and plated into 15 ml of incubation medium. Radioactive orthophosphate (8 mCi, final specific activity in the medium 4 Ci/mmol) and [*3,5*-^{3}H]tyrosine (75 μCi, final specific activity in the medium 250 Ci/mol) were added. The chunks were incubated on a rocker for 9–10 hr in a humidified 5% CO_2 atmosphere. The incubation was stopped by washing the chunks three times in chilled complete L-15 medium. Cold carrier rat brain (1–1.5 g wet weight) was added, and microtubule protein was purified as described above. Labeled microtubule protein was prepared from slices of adult rat brain by the same procedure.

Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide gels (7.5% acrylamide–0.27% methylenebisacrylamide) were run as described by Weber and Osborn (7) with either phos-

* Tissue culture medium was made by the method of Richard Mains, personal communication.

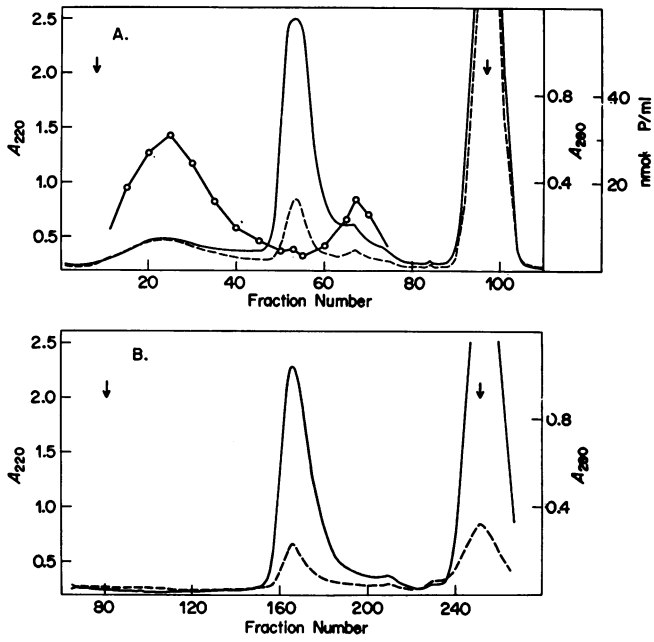

FIG. 1. (*A*) Microtubule protein was purified by the method of Weisenberg *et al.* (5), except that ion-exchange chromatography was done on DEAE-cellulose instead of DEAE-Sephadex A-50. Peak fractions were pooled, and the protein was precipitated by addition of HCl to pH 4. The pellet was dissolved in 5% SDS–5% 2-mercaptoethanol–25 mM Tris·HCl (pH 7.8) by boiling for 2 min. Gel filtration was done on a column of Sepharose 4B in 0.04 M Tris·H_2SO_4–0.2% SDS (pH 8.0). *Arrows* mark the void volume and the column volume. (*B*) Microtubule protein was purified by the pyrophosphate procedure described. Peak fractions from the DEAE-cellulose column were pooled and precipitated with HCl. The pellet was dissolved in 5% SDS–2.5% 2-mercaptoethanol–0.1 M Tris·HCl (pH 7.3) by incubation at 40° for 4 hr. Column conditions were exactly the same as in *A*. Absorbance at 220 nm (——); absorbance at 280 nm (– – –); nmol P/ml (O——O).

phate buffer or the borate acetate buffer of Davies and Stark (8). Urea–SDS gels were made according to Davis (9) with the inclusion of 8 M urea and 0.1% SDS; the reservoir buffer contained 0.01% SDS. Discontinuous 8 M urea gels (10% acrylamide–0.15% methylenebisacrylamide) were run at pH 4.5; the reservoir buffer was 0.07 M β-alanine–acetic acid. Gels were stained with Coomassie brilliant blue and destained by diffusion in 42.5% (v/v) methanol–7.5% (v/v) acetic acid. Gels of radioactively labeled microtubule protein were analyzed by slicing the gels into 1- or 2-mm discs, digesting the slices in 0.2 ml of 30% hydrogen peroxide (10), and counting in 4 ml of Aquasol (New England Nuclear). Radioactive protein was recovered from gels by cutting out the region of proper mobility from an unstained gel and transferring the protein by electrophoresis into a dialysis bag as described by Feit *et al.* (11). Microtubule protein was reduced and alkylated by the method of Renaud (12); unless stated otherwise, alkylations were done at pH 8.8. The extent of reaction was determined by amino-acid analysis.

Phosphate and Sugar Assays. Amino sugars were separated on the short column of a Beckman model 120C amino-acid analyzer with pH 4.25 citrate buffer. Hydrolysis for amino sugars was done in 3 N HCl for 6, 12, or 20 hr with β-alanine as an internal standard. Neutral sugars were assayed by the phenol–sulfuric acid method of Dubois (13) with mannose, galactose, and ribose as standards. Phosphate was determined by the method of Ames and Dubin (14). Protein was determined by amino-acid analysis performed by the method of Spackman *et al.* (15).

Enzymatic Digestions. Digestions with Pronase (Calbiochem B grade) were done in 0.1 M Tris·HCl–2 mM $CaCl_2$ (pH 7.45) at 37° for 2 hr at an enzyme to substrate ratio of 1:11. Subtilisin (Sigma Type VII) digestions were done in 0.05 M ammonium bicarbonate at 37° for 2 hr at an enzyme to substrate ratio of 1:20. Ribonuclease (Worthington pancreatic ribonuclease A) digestions were done in PPMg at 37° for 1 hr at a ratio of 1:17. Deoxyribonuclease (Worthington 2400 U/mg) was used in PPMg at a ratio of 1:20 for 2 hr at 37°. Venom phosphodiesterase (Worthington) was used for 30 min at 37° in a 1:20 ratio in 0.1 M Tris·HCl–5.0 mM $MgCl_2$ (pH 9.0); 2 mM phenylmethanesulfonylfluoride (16) was included to inhibit proteolytic activity present in the enzyme. Microtubule protein was freed of inorganic phosphates by passage through a Sephadex G-50 column in 0.05 M Tris·HCl–2.5 mM $MgCl_2$ (pH 8.0). Alkaline phosphatase (Sigma Type III-S, 10 U/mg) was added in a ratio of 1:20, and digestions were done at 37° for 2 hr. Potato acid phosphatase (Sigma Type II) was used at a 1:10 ratio in 0.15 M sodium acetate (pH 5.5) at 37° for 2 hr.

RESULTS

Microtubule protein prepared from rat brain by Procedure 1 of Weisenberg, Borisy, and Taylor (5) is about 95% pure,

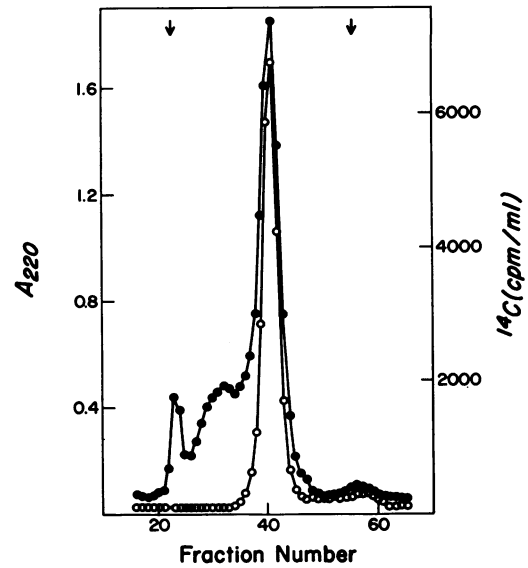

FIG. 2. The 35–50% ammonium sulfate cut was resuspended in PPMg and incubated with [*methoxy*-^{14}C]colchicine (4.2 μM, 8.1 Ci/mol) at 37° for 1 hr. The DEAE-cellulose column was run and the fractions containing microtubule protein were pooled, concentrated by vacuum dialysis, and run on a Bio-Gel A-1.5m column in PPMg plus 0.1 M KCl–0.1 mM dithiothreitol (pH 7.0). The colchicine-binding activity elutes at a position between rabbit muscle aldolase and bovine serum albumin. The material around *fraction 32* is aggregated microtubule protein; the amount of aggregate can be decreased greatly if the sample is not concentrated before application to the column. Absorbance at 220 nm (●——●; [^{14}C]colchicine (cpm/ml) (O——O).

as judged by scans of polyacrylamide gels run in SDS or urea and stained with Coomassie brilliant blue. The protein appears to contain phosphate and carbohydrate. The ratio of absorbance at 280 nm to absorbance at 260 nm decreases through the peak of microtubule protein, and the amount of phosphate and carbohydrate per mol of protein dimer increases through the peak. Microtubule protein purified on DEAE-cellulose columns in phosphate buffers contains up to 10 mol of organic phosphate per mol of dimer, 10 mol of neutral sugar (calibrated as ribose) per mol of dimer, 4 mol of galactosamine, and 4 mol of glucosamine per mol of dimer. Gel filtration on Sepharose 4B in 0.2% SDS resolves microtubule protein from materials that are similar to nucleic acids in their spectral and chemical properties (Fig. 1*A*). Microtubule protein purified on DEAE-cellulose in pyrophosphate buffers as described above contains much less of these contaminants (Fig. 1*B*). The traces of nucleic acid remaining can be removed by gel filtration of native microtubule protein on Bio-Gel A-1.5m (Fig. 2). Based on recovery of colchicine binding activity, the pyrophosphate method gives yields of 25–30% of the starting soluble activity; one can prepare 1 mg of DEAE-purified microtubule protein from 1 g (wet weight) of starting tissue.

Reduced and carboxamidomethylated rat brain microtubule protein shows two widely spaced bands on SDS-urea gels (with mobilities of 0.48-0.49 and 0.53-0.54). Electrophoretically distinguishable subunits have been observed in microtubule protein isolated from chick embryo brain (17), cultured chick sympathetic neurons (18), pig brain (11, 19), mouse brain (11), and neuroblastoma (11, 19). The subunits will be referred to as α and β, with β the more rapidly migrating of the two components on SDS-urea gels. The bands do not stain with equal intensity (Fig. 3*A*), but amino-acid analysis of gel slices shows that the two are present in equimolar amounts (Table 1) and that they differ significantly in amino-acid composition (Table 2). These results agree with those of Bryan and Wilson (17), Feit *et al.* (11), and Fine (18). When iodoacetic acid is used as the alkylating agent instead of iodoacetamide, two barely resolved bands are observed (Fig. 3*B*) (mobilities 0.47 and 0.49). Therefore, mobility in this system not only reflects polypeptide size, it also depends on the charge on the polypeptide chain. SDS gels show no indication of two components of different size, so there is no reason to assume that the subunits of rat brain microtubule protein differ by more than 2000 in molecular weight.

Microtubule protein purified as described above contains no detectable amino sugars (glucosamine less than 0.07 mol/mol of dimer, galactosamine less than 0.01 mol/mol of dimer), no more than 1.2 mol of neutral sugar (calibrated as galactose) per mol of dimer, and 0.8 ± 0.2 mol of covalently bound phosphate per mol of dimer (Table 3). The occurrence of neutral sugars without amino sugars has been reported only in collagens and basement membranes (20), so it is not yet

TABLE 1. *Quantitation of protein in α and β chains*

Conditions of alkylation (pH)	Number of gels pooled	nmol of amino acids		Ratio α/β
		α	β	
9.5	3	159	165	0.96
9.5	2	132	134	0.98
8.8	3	190	201	0.94
8.8	2	124	115	1.08
Mean and standard deviation				0.99 ± 0.05

Bands were cut from stained gels and hydrolyzed in 6 N HCl for 24 hr at 110°. Norleucine was included as an internal standard. Calculations are based only on the nine amino acids that are determined accurately after hydrolysis of stained gel slices (23): Asp, Thr, Glu, Pro, Ala, Val, Ile, Leu, Phe (tyrosine was not included because values were consistently low).

TABLE 2. *Partial amino-acid analysis of subunits*

Amino acid	Mol/55,000 g	
	α	β
Aspartic acid	50.5 (3.8)	54.9 (1.3)
Threonine	29.2 (1.7)	31.8 (0.6)
Serine*	25.0 (1.5)	31.5 (1.7)
Glutamic acid	60.0 (1.6)	65.5 (2.2)
Proline	22.8 (3.3)	24.6 (3.5)
Glycine	46.5 (2.0)	44.8 (1.7)
Alanine*	39.0 (2.0)	32.1 (2.3)
Valine	32.6 (0.9)	30.6 (2.6)
Isoleucine*	29.7 (2.2)	22.0 (1.7)
Leucine	31.3 (2.9)	31.2 (2.2)
Phenylalanine	19.6 (0.9)	21.8 (0.2)

Amino-acid analyses were performed as described in Table 1. The sum of the nine amino acids listed in Table 1 was adjusted to equal the sum of these nine amino acids from a proper hydrolysate of rat brain microtubule protein (unpublished data). Serine and glycine were calculated in relation to these nine amino acids. Serine values obtained here were not significantly higher than expected from the amino-acid composition of rat brain microtubule protein. Glycine values are high because of background from the gel slice. Valine values are low because the numbers given are from a 24-hr hydrolysate only. Values given are the average of four determinations; the maximum deviation is given in parentheses. Significant differences are marked with *.

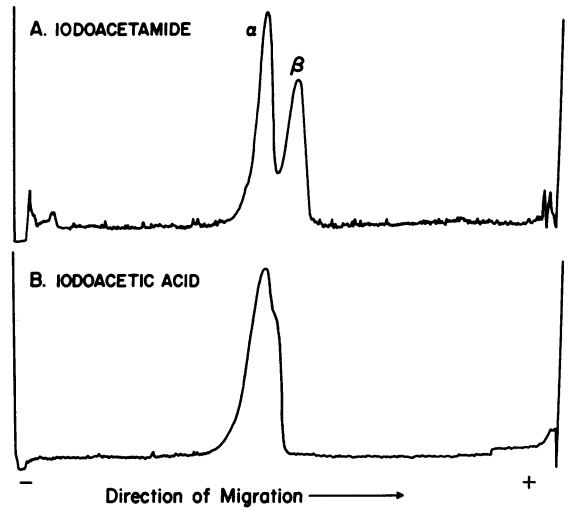

FIG. 3. (*A*) Microtubule protein was reduced and alkylated with iodoacetamide and run on SDS–urea gels. Gels were scanned at 604 nm on a Zeiss spectrophotometer with linear transport attachment. Scans include the region of the gel from the top of the running gel down to the dye band. (*B*) Same as *A* except microtubule protein was alkylated with iodoacetic acid.

TABLE 3. *Quantitation of phosphate, neutral sugar, and amino sugar bound to microtubule protein*

Sample preparation*	Mol/mol of dimer (110,000 g)			
	Phosphate	Neutral sugar (galactose)	Amino sugars: Glucosamine	Amino sugars: Galactosamine
1. DE52 in PPMg, A-1.5m, desalted on Bio-Gel P10	0.6	0.4	—	—
2. DE52 in PPMg, A-1.5 m, desalted on Bio-Gel P10	0.6	0.6	—	—
3. DE52 in PPMg, Sepharose 4B	1.0	3.2	0.07	0.01
4. DE52 in PPMg, Sepharose 4B	0.4	0.7	—	—
5. DE52 in phosphate buffer, Sepharose 4B	1.1	1.1	—	—
6. Rerun of microtubule protein peak from no. 5 on Sepharose 4B	0.9	—	0.03	0
Mean ± SD	0.8 ± 0.2	1.2 ± 1.0		

* The eluant used for the Bio-Gel P10 and the Sepharose 4B columns contained 0.2% SDS; GTP is separated from the protein by this method.

clear whether or not the low and variable amounts of neutral sugar observed are due to sugar covalently bound to the protein. In order to distinguish between phosphate covalently bound to microtubule protein and the presence of a small amount of a phosphate containing contaminant, ^{32}P-labeled microtubule protein was prepared.

Brain slices from neonatal or adult rats were incubated with [^{3}H]tyrosine and [^{32}P]orthophosphate as described above. The [^{3}H]tyrosine was included to mark the position of the protein and to check the viability of the slices. Labeled microtubule protein purified through the DEAE-cellulose step was denatured in SDS and run on polyacrylamide gels in SDS at pH 7.2 and at pH 8.5. A peak of phosphate counts coincides with the peak of tritium counts that has the mobility of microtubule protein (Fig. 4*A*). The ^{32}P counts are also coincident with microtubule protein when run on urea gels at pH 4.5 (Fig. 4*B*). When labeled reduced and alkylated microtubule protein is run on SDS-urea gels, peaks of [^{3}H]-tyrosine counts are observed at the mobility of the α and β subunits. Only the β chain has ^{32}P counts (Fig. 4*C*). Thus, native microtubule protein is phosphorylated specifically on only one of the two subunits. The ratio of ^{3}H to ^{32}P counts in the microtubule protein peak is the same when samples are prepared by any of the three following procedures: (*i*) addition of 2% SDS followed by incubation at room temperature for 30 min; (*ii*) acid precipitation of microtubule protein, addition of 2% SDS and 1% 2-mercaptoethanol, and incubation for 30 min at room temperature; or (*iii*) acid precipitation of microtubule protein, addition of 2% SDS and 1% 2-mercaptoethanol, and incubation in a boiling-water bath for 2 min. Digestion with Pronase or subtilisin removes completely both the ^{3}H and ^{32}P counts from the microtubule region of the gel. Digestion with ribonuclease, deoxyribonuclease, or venom phosphodiesterase does not alter the ratio of ^{3}H to ^{32}P counts in the microtubule protein peak. Lipid extraction (21) and base treatment (0.1 N sodium hydroxide, 2 hr, room temperature) do not alter the ratio. Neither alkaline phosphatase nor potato acid phosphatase will remove the bound [^{32}P]phosphate.

SDS gels of DEAE-purified labeled microtubule protein from newborn rat brains show protein-bound phosphate counts on polypeptide chains other than microtubule protein

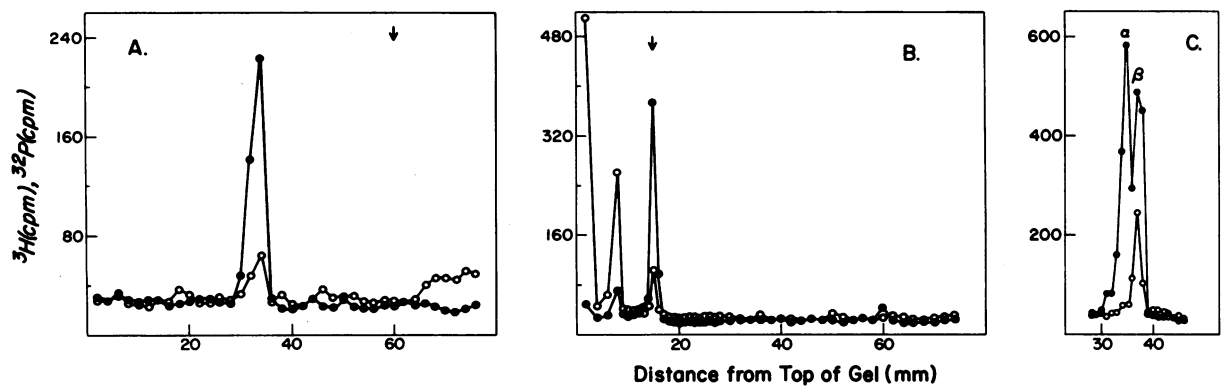

FIG. 4. (*A*) Labeled microtubule protein purified through the DEAE-column step was precipitated with HCl and dissolved in 1% SDS–1% 2-mercaptoethanol–50 mM borate–50 mM sodium acetate (pH 8.5). Gels were run in pH 8.5 borate–acetate buffer at 5 m*A*/gel for 4 hr. *Arrow* marks the position of the tracker dye. (*B*) Labeled DEAE-purified microtubule protein from baby rat brain was reduced and alkylated with iodoacetamide and run on pH 4.5 8 M urea gels at 2.5 mA/gel for 3 hr. The ^{32}P-labeled contaminants in this preparation of microtubule protein are present at the top of the stacking gel and at the interface between the stacking gel and the running gel. *Arrow* marks the position of microtubule protein. (*C*) Labeled DEAE-purified microtubule protein was reduced and alkylated with iodoacetamide. The sample was made 0.1% in SDS and run on SDS–urea gels at 1 mA/gel for 5 hr. ^{3}H (cpm) (●——●); ^{32}P (cpm) (○——○).

Scans of stained gels show that these contaminating proteins are present in very small amounts; they are not significantly labeled with the marker amino acid and, therefore, must not turn over rapidly compared to microtubule protein. These impurities are present in labeled microtubule protein prepared from adult brain in greatly reduced amounts, and gel filtration on Bio-Gel A-1.5m resolves them completely from microtubule protein. Labeled microtubule protein prepared from baby rat brains was used for the identification of the phosphorylated amino acid and was purified by preparative SDS gel electrophoresis. After partial acid hydrolysis (2 N HCl, 100°, 2 hr) of microtubule protein, phosphate counts migrated either as free orthophosphate or with unlabeled carrier serine phosphate in each of the two following systems: (*i*) electrophoresis on paper at pH 3.44 (10% acetic acid-1% pyridine) 1500 V for 140 min; and (*ii*) electrophoresis on paper at pH 5.30 (6% pyridine-2.85% acetic acid) 1500 V for 135 min followed by chromatography in isobutyric acid-0.5 N ammonia (10:6) (22). Both systems resolve serine phosphate from threonine phosphate. Rat brain microtubule protein is phosphorylated on a serine residue in the β chain.

DISCUSSION

Rat brain microtubule protein contains no covalently bound amino sugars and no more than 0.2% neutral sugar. The finding of amino sugars (4) and larger amounts of neutral sugars (2, 3) by other investigators may be due to the presence of contaminating nucleic acids and carbohydrates. It has been found, however, that native microtubule protein isolated from the soluble fraction of rat brain homogenates is phosphorylated specifically on the β chain. In rat brain microtubule protein, as in all other neural microtubule proteins studied, there are two electrophoretically distinct subunits. The α and β chains of rat brain microtubule protein do not differ simply by the presence and absence of phosphate; they also differ in amino-acid composition in much the same way as the chick brain microtubule protein subunits studied by Bryan and Wilson (17).

One might argue that the phosphorylation observed here is the result of a phosphorylation catalyzed by a protein kinase that occurs during purification of the protein, and that microtubule protein in intact cells is not phosphorylated. This seems unlikely, since phosphorylation is specific to one subunit, and is approximately stoichiometric (0.8 ± 0.2 mol/mol of dimer). It is not yet possible to compare the phosphorylation observed here with the cAMP-stimulated phosphorylation of bovine brain microtubule protein observed by Goodman *et al.* (2), or the phosphorylation of rat brain microtubule protein catalyzed by hog brain kinase studied by Murray and Froscio (1). Neither Goodman *et al.* (2) nor Murray and Froscio (1) reported the stoichiometry of the phosphorylation, and it is not known whether the microtubule proteins were phosphorylated on only one subunit. The phosphorylation observed here may occur by a process unrelated to the above mentioned phosphorylation of microtubule protein by cAMP-stimulated protein kinases.

The major question now is whether phosphorylation of microtubule protein plays a role in the cellular control of microtubule function. Goodman *et al.* (2) discussed the possible role of phosphorylation of microtubule protein in neurosecretion. Covalent modification of the microtubule protein subunits might also be involved in control of assembly and disassembly of microtubules. The ability of microtubule protein dimers to polymerize could be regulated by the state of phosphorylation of the protein subunits. A mechanism such as this would allow rapid response of the microtubule system to specific cellular events through activation of the enzymes responsible for the phosphorylation and dephosphorylation of microtubule protein. In order to evaluate the possible involvement of phosphorylation in control of assembly of microtubules, it is essential to determine whether intact microtubules, microtubule protein that is associated with the particulate fraction of cell homogenates, and microtubule protein free in the cytoplasm are characterized by different levels of phosphorylation.†

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† The microtubule protein studied here may arise from any of these three pools of microtubule protein.