Conservation of Microtubule Associated Proteins

ISOLATION AND CHARACTERIZATION OF \( \tau \) AND THE HIGH MOLECULAR WEIGHT MICROTUBULE ASSOCIATED PROTEIN FROM CHICKEN BRAIN AND FROM MOUSE FIBROBLASTS AND COMPARISON TO THE CORRESPONDING MAMMALIAN BRAIN PROTEINS*

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Using ability to stimulate tubulin assembly as an assay, we have purified a chicken brain associated protein under conditions identical to those used to isolate porcine brain \( \tau \) protein. Chicken brain \( \tau \) has a molecular weight, sedimentation coefficient, and amino acid composition very similar to porcine \( \tau \). Both \( \tau \) proteins are microheterogeneous and yield very similar and characteristic one-dimensional peptide maps. We have also isolated \( \tau \) and a high molecular weight protein from simian virus 40-transformed 3T3 cells by copolymerization of labeled cellular proteins with carrier hog brain microtubules.

The simian virus 3T3 cell proteins co-electrophorese with the corresponding hog brain associated proteins and yield peptide maps which are indistinguishable from the hog protein patterns. These findings confirm the presence of \( \tau \) and a high molecular weight microtubule associated protein from a nonneuronal source and demonstrate that both are present in a single cell type, as originally suggested by immunofluorescent localization with antibodies against the hog brain proteins. Moreover, the similarities of the \( \tau \) polypeptides from the three sources studied (hog brain, chicken brain, and mouse fibroblast) imply that \( \tau \) is a widely distributed and conserved microtubule protein. The similarities between the high molecular weight associated proteins from hog brain and mouse fibroblast suggest that they, too, are widely distributed.

Although proteins co-purifying with tubulin in microtubules have been demonstrated in a number of tissues and species, only in microtubules purified from mammalian brain have individual associated proteins been purified using their ability to induce microtubule assembly as an assay (Cleveland et al., 1977b; Murphy et al., 1977; Fellous et al., 1977; Herzog and Weber, 1978b; Kuznetsov et al., 1974; Kim et al., 1979). It is only from hog brain that they have been characterized by a number of physical and chemical properties (Cleveland et al., 1977c; Murphy et al., 1977; Herzog and Weber, 1978b). Tubulin, on the other hand, has been purified from a number of species and shown to possess a rather high conservation of physical and chemical properties (see, for example, reviews by Snyder and McIntosh, 1976; Stephens and Eldes, 1976; Kirschner, 1978). Consequently, we wished to determine, first, whether microtubule associated proteins of the type purified from hog brain are also widely distributed both phyleogenetically and in various cell types and, second, whether these proteins have diverged widely in their physical and chemical properties. Although there may well be other proteins which are present in microtubules from brain and other tissues, we have confined ourselves initially to look for \( \tau \) and HMW (i.e., the high molecular weight microtubule associated protein), the two associated proteins which are best characterized and which have been detected in microtubules by immunocytochemical methods (Connolly et al., 1977, 1978; Sherline and Schiavone, 1977, 1978).

MATERIALS AND METHODS

Preparation of Microtubule Protein, Purified Tubulin, and \( \tau \)—Microtubule protein was prepared from porcine or chicken brain by the Weingarten et al. (1974) modification of the method of Shelanski et al. (1974). Phosphocellulose-purified tubulin was prepared by chromatography of microtubule protein as described by Weingarten et al. (1975). After removal of the tubulin, a crude associated protein fraction was obtained by elution of the adsorbed protein with 1.0 M NaCl. This fraction then was desalted on Bio-Gel P6 into Buffer P (0.1 M 2-(N-morpholino)ethanesulfonic acid (pH 6.4), 1 mM ethylene glycol bis(2-aminoethyl ether)N,N'-tetraacetic acid, 0.1 mM EDTA, 0.5 mM MgCl₂, and 1 mM 2-mercaptoethanol). Hog \( \tau \) was further purified as previously described (Cleveland et al., 1977b) by a combination of phosphocellulose chromatography, ammonium sulfate precipitation, and hydroxypatite chromatography. Chicken \( \tau \) was purified in an analogous fashion with the exceptions noted in the text.

The ability of associated protein fractions to stimulate assembly of phosphocellulose-purified tubulin was monitored by quantitative electron microscopy as initially described by Kirschner et al. (1975).

Protein concentrations were determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard. Hydroxyapatite was purchased from Bio-Rad. Ammonium sulfate (ultrapure) was obtained from Schwarz/Mann and phosphocellulose (P11, batch 359) was from Whatman.

Polyacrylamide Gel Electrophoresis—Polyacrylamide gel electrophoresis was performed on slab gels according to the method of Laemmli (1970) with the resolving gel buffer adjusted to pH 9.1. Gels were stained immediately after electrophoresis by the method of Shelanski et al. (1974) modification of the method of Laemmli (1970) with the resolving gel buffer adjusted to pH 9.1. Gels were stained immediately after electrophoresis in a solution of 0.25% Coomassie blue R250, 45% methanol, 9% acetic acid. Destaining was achieved by diffusion in a solution of 5% methanol, 7.5% acetic acid.

Peptide Mapping—One-dimensional peptide mapping of hog and chicken proteins isolated from polyacrylamide gels was performed by "proteolysis during re-electrophoresis" in the presence of SDS as described by Cleveland et al. (1977a). Samples were initially radioactively labeled in vitro with [³⁵S]methionine by the reductive methylation technique of Rice and Means (1971). Individual protein bands were excised from a preparative SDS-polyacrylamide gel after

* The abbreviations used are: SDS, sodium dodecyl sulfate; SV, simian virus; Pipes, 1,4-piperazinediethanesulfonic acid; Mes, 2-(N-morpholino)ethanesulfonic acid.
apatite column equilibrated in Buffer P. Adsorbed protein was eluted with 45% ammonium sulfate. Precipitated protein (i.e., the r fraction) was collected by centrifugation and will be referred to as the 38 to 45% protein fraction. This 38 to 45% fraction then was chromatographed on an hydroxyapatite column which then was eluted with 0.15 M phosphate in Buffer P. Tubulin from hog and chicken was precipitated in a single 0.35 M NaCl wash of phosphocellulose to which had been applied microtubule protein purified through one, two, and three cycles of assembly; Lane 5, purified tubulin obtained by chromatography of two-cycle microtubule protein on phosphocellulose; Lane 6, total microtubule accessory proteins obtained with a 1.0 M NaCl wash of phosphocellulose to which had been applied two-cycle microtubule protein; Lane 7, initial crude chicken r fraction prepared as in Lane 6 except that a 0.35 M NaCl wash was employed; Lane 8, microtubule-associated proteins which eluted in a 1.0 M NaCl wash following the 0.35 M NaCl wash in Lane 7; Lane 9, protein eluted from phosphocellulose in the 0.35 M NaCl wash and which precipitated in 38% saturated ammonium sulfate; Lane 10, protein eluted from phosphocellulose in the 0.35 M NaCl wash and which precipitated in 38% saturated ammonium sulfate; Lane 11, purified chicken r obtained by application of the 38 to 45% ammonium sulfate-precipitable protein to an hydroxyapatite column which then was eluted with 0.15 M phosphate in Buffer P; Lane 12, protein eluted in 0.5 M phosphate from the hydroxyapatite column of Lane 11. Molecular weight standards are shown in units at 1000 to the left of the figure, with a- and β-tubulin taken to be 55,000 and 53,000, respectively.

**Fig. 1.** Assay of crude associated protein fractions from hog and chicken brain for stimulation of polymerization of hog and chicken tubulin. Crude associated protein fractions from both hog and chicken were obtained by elution with 1.0 M NaCl from phosphocellulose to which had been applied microtubule protein purified by two cycles of assembly. Recovered associated proteins were desalted into Buffer P. Tubulin from hog and chicken was prepared by chromatography on phosphocellulose as previously described (Weingarten et al., 1975) and was used within 3 h of elution from phosphocellulose. Assays for polymerization were performed by quantitative electron microscopy. Final hog and chicken tubulin concentrations were 1.17 and 1.27 mg/ml, respectively. A, levels of polymerization at constant initial concentrations of hog tubulin (○—○) and chicken tubulin (●—●) with increasing amounts of added crude chicken associated proteins. B, levels of polymerization of hog tubulin (□—□) and chicken tubulin (●—●) with increasing amounts of added crude hog associated proteins.

**Fig. 2.** Assay of fractions from the chicken r purification by quantitative electron microscopy. Total associated proteins were prepared by elution with a 1.0 M NaCl wash of phosphocellulose to which had been applied microtubule protein purified by two cycles of assembly. The r purification was begun with a 0.35 M NaCl wash of phosphocellulose. After desalting into Buffer P, this r fraction was brought to 38% saturated ammonium sulfate. Precipitated protein was removed by centrifugation and the supernatant was brought to 45% ammonium sulfate. Precipitated protein (i.e., the r fraction) was collected by centrifugation and will be referred to as 38 to 45% protein. This 38 to 45% fraction then was chromatographed on an hydroxyapatite column which then was eluted with 0.15 M phosphate in Buffer P; Lane 11, purified chicken r obtained by application of the 38 to 45% ammonium sulfate-precipitable protein to an hydroxyapatite column which then was eluted with 0.15 M phosphate in Buffer P; Lane 12, protein eluted in 0.5 M phosphate from the hydroxyapatite column of Lane 11. Molecular weight standards are shown in units at 1000 to the left of the figure, with a- and β-tubulin taken to be 55,000 and 53,000, respectively.

**Fig. 3.** SDS/polyacrylamide gel electrophoresis of fractions from the purification of r from chicken brain. The resolving gel was cast from 8.5% acrylamide. Lane 1, chicken brain extract; Lanes 2 to 4, microtubule protein purified through one, two, and three cycles of assembly; Lane 5, purified tubulin obtained by chromatography of two-cycle microtubule protein on phosphocellulose; Lane 6, total microtubule accessory proteins obtained with a 1.0 M NaCl wash of phosphocellulose to which had been applied two-cycle microtubule proteins; Lane 7, initial crude chicken r fraction prepared as in Lane 6 except that a 0.35 M NaCl wash was employed; Lane 8, microtubule-associated proteins which eluted in a 1.0 M NaCl wash following the 0.35 M NaCl wash in Lane 7; Lane 9, protein eluted from phosphocellulose in the 0.35 M NaCl wash and which precipitated in 38% saturated ammonium sulfate; Lane 10, protein eluted from phosphocellulose in the 0.35 M NaCl wash and which precipitated in 38% saturated ammonium sulfate; Lane 11, purified chicken r obtained by application of the 38 to 45% ammonium sulfate-precipitable protein to an hydroxyapatite column which then was eluted with 0.15 M phosphate in Buffer P; Lane 12, protein eluted in 0.5 M phosphate from the hydroxyapatite column of Lane 11. Molecular weight standards are shown in units at 1000 to the left of the figure, with a- and β-tubulin taken to be 55,000 and 53,000, respectively.
relative to that of the hog proteins in SDS-polyacrylamide gels. SDS-polyacrylamide gels of the final co-polymerization product obtained from phosphocellulose chromatography were briefly stained and destained and the hog and SV3T3 \( \tau \) and HMW bands were excised. These bands were crushed in a glass homogenizer with a Teflon pestle in 0.5 ml of 0.125 M Tris (pH 6.8), 0.1% SDS. This slurry was incubated at 37°C for 15 h, and, following centrifugation, proteins in the supernatant were precipitated by the addition of 20% trichloroacetic acid and 50 \( \mu \)g of tubulin. The precipitated protein was collected by centrifugation, extracted with ether, and dissolved in 0.5 ml of 0.125 M Tris (pH 6.8), 0.5% SDS, and 1 mM EDTA. Proteolysis in solution was subsequently performed as described (Cleveland et al., 1977a).

**Results**

Preparation of Microtubule Associated Proteins from

![FIG. 4. Sucrose gradient centrifugation of chicken \( \tau \).](image)

**FIG. 4.** Sucrose gradient centrifugation of chicken \( \tau \). Chicken \( \tau \) was applied at 0.5 mg/ml to an 11 ml 5 to 25% sucrose gradient in Buffer P and spun in an SW 41 rotor at 4°C at 39,000 rpm for 18 h. Gradients loaded with proteins of known sedimentation coefficients were run in parallel. Gradients were collected and the \( \tau \) gradient was analyzed by absorbance profile and by ability to stimulate polymerization in phosphocellulose purified hog tubulin. Assays of polymerization were formed by quantitative electron microscopy at a final tubulin concentration of 1.1 mg/ml. ○ ○ ○, absorbance profile; □ □ □, positions and \( S_m \), values of marker proteins run in parallel gradients (from left to right, squares indicate: Escherichia coli \( \beta \)-galactosidase, beef liver catalase, E. coli alkaline phosphatase, bovine serum albumin, and horse heart cytochrome c).

**TABLE 1**

Amino acid compositions (mol %) of \( \tau \) from chicken and from hog brain

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Chicken ( \tau )</th>
<th>Hog ( \tau )</th>
<th>Hog HMW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>9.7</td>
<td>9.8</td>
<td>9.7</td>
</tr>
<tr>
<td>His</td>
<td>2.8</td>
<td>2.9</td>
<td>2.6</td>
</tr>
<tr>
<td>Arg</td>
<td>4.0</td>
<td>3.9</td>
<td>3.6</td>
</tr>
<tr>
<td>Asp</td>
<td>8.9</td>
<td>8.9</td>
<td>9.7</td>
</tr>
<tr>
<td>Thr</td>
<td>6.9</td>
<td>6.7</td>
<td>7.6</td>
</tr>
<tr>
<td>Ser</td>
<td>10.0</td>
<td>9.5</td>
<td>10.1</td>
</tr>
<tr>
<td>Glu</td>
<td>13.1</td>
<td>12.5</td>
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<tr>
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<td>10.4</td>
</tr>
<tr>
<td>Gly</td>
<td>10.3</td>
<td>9.4</td>
<td>10.9</td>
</tr>
<tr>
<td>Ala</td>
<td>9.0</td>
<td>9.0</td>
<td>7.9</td>
</tr>
<tr>
<td>Val</td>
<td>5.0</td>
<td>5.0</td>
<td>5.1</td>
</tr>
<tr>
<td>Met</td>
<td>0.7</td>
<td>0.6</td>
<td>1.2</td>
</tr>
<tr>
<td>Ile</td>
<td>4.1</td>
<td>4.1</td>
<td>5.5</td>
</tr>
<tr>
<td>Leu</td>
<td>4.5</td>
<td>4.5</td>
<td>5.5</td>
</tr>
<tr>
<td>Tyr</td>
<td>1.0</td>
<td>1.6</td>
<td>1.0</td>
</tr>
<tr>
<td>Phe</td>
<td>0.9</td>
<td>1.2</td>
<td>0.9</td>
</tr>
</tbody>
</table>

* Hydroxyapatite-purified chicken \( \tau \) further purified by electrophoresis on an 8.5% gel prior to hydrolysis.

**FIG. 5. Isolated HMW and \( \tau \) polypeptides rerun on an 8.5% gel to verify their purity.** Lane 1, the 0.35 M NaCl wash of phosphocellulose from which the hog HMW and hog \( \tau \) bands were excised; Lane 2, isolated hog HMW protein; Lane 3, isolated higher molecular weight hog \( \tau \) doublet; Lane 4, isolated lower hog \( \tau \) doublet; Lane 5, the 0.35 M NaCl wash of phosphocellulose to which was applied two-cycle chicken microtubule protein; Lane 6, isolated chicken HMW protein as excised from the protein shown in Lane 5; Lane 7, chicken \( \tau \) purified through ammonium sulfate fractionation and the sample from which the individual chicken \( \tau \) bands were excised; Lanes 8 to 11, the isolated chicken \( \tau \) bands of highest (Lane 8) through lowest (Lane 11) molecular weight. Note the presence of some apparent dimer and trimerization of the samples in Lanes 9 and 10. These can be avoided by incubating the gel slices in 0.1% mercaptoethanol prior to placing on the second gel.

**Amino Acid Analysis**—For amino acid analysis, 10 to 20 \( \mu \)g of each sample (isolated from a preparative 8.5% SDS-polyacrylamide gel or purified by conventional biochemical methods) was hydrolyzed in constant boiling HCl (Pierce) by incubation at 108°C for 24 h and then applied to a Durrum amino acid analyzer equipped with a numerical integrator for measurement of peak areas.

**Cell Culture and Radiolabeling**—SVT2, an SV40-transformed Balb/c 3T3 cell line was provided by Dr. A. J. Levine, Princeton University. They were grown at 37°C in Dulbecco’s modified Eagle’s medium containing 10% calf serum in a 10% CO2 atmosphere. Cells were subcultured by trypsinization and 5 x 105 cells were placed in 100-mm plastic Petri dishes in Dulbecco’s modified Eagle’s medium. After 24 h this medium was replaced with 10 ml of methionine-free Dulbecco’s modified Eagle’s medium supplemented with 30 \( \mu \)g of methionine and 1.0 mCi of [\( ^35 \)S]methionine (100 to 400 Ci/mmol, NEG-009H; New England Nuclear).

**Purification of Labeled SV3T3 Extracts by Co-polymerization with Porcine Microtubule Protein**—After the cells were labeled for 24 to 36 h in three 100 mm Petri dishes, the radioactive medium was removed. The cells were rinsed with ice-cold phosphate-buffered saline and harvested in a small volume of phosphate-buffered saline by scraping the surface of the dish with a rubber serum stopper. The cells were collected by centrifugation and homogenized in 1.0 ml of Buffer P in a glass homogenizer with a motor-driven Teflon pestle. Cell nuclei and large debris were removed by centrifugation at 12,000 x g for 10 min at 4°C. Radioactive microtubule proteins were purified from these extracts by co-polymerization in the absence of glycerol, followed by phosphocellulose chromatography as described by Spiegelman et al. (1977), with the exception that the salt purification step prior to the first cycle of assembly was omitted.
t and HMW Are Conserved Microtubule Associated Proteins

Fig. 6. Peptide maps of isolated chicken and hog \( \tau \) bands and of the chicken and hog HMW polypeptides. Individual chicken and hog \( \tau \) and HMW proteins were isolated on an initial 8.5% SDS-polyacrylamide gel. Peptide maps on a 18% gel then were produced using chymotrypsin as detailed under “Materials and Methods.” A fluorograph of the resultant peptide pattern is shown. Slots 1 to 3 of each triplet were protein loaded with 0, 2, and 8 \( \mu g \) of chymotrypsin, respectively. A, hog HMW; B, chicken HMW protein; C, the two higher molecular weight hog \( \tau \) bands, isolated as a doublet; D, the two lower molecular weight hog \( \tau \) bands isolated as a doublet; E to H, the four chicken \( \tau \) bands of highest (E) through lowest (H) molecular weight. All slots in A through H are from the same 15% gel.

Chicken Brain—Microtubule protein was prepared from adult chicken brains by two cycles of temperature-dependent assembly/disassembly as described under “Materials and Methods.” Associated proteins then were separated from the tubulin by chromatography on phosphocellulose. After three cycles of assembly, the associated protein fraction comprised about 15% of the total protein. The total associated protein fraction was recovered in a single 1.0 M NaCl wash and, following desalting into Buffer P, was assayed for ability to stimulate polymerization of phosphocellulose purified tubulin. The level of microtubule assembly using hog or chicken tubulin and increasing levels of crude chicken accessory protein was assayed by quantitative electron microscopy. Crude chicken associated proteins stimulate comparable levels of polymerization in either chicken or hog tubulin (Fig. 1A). The extent of polymerization is proportional to the amount of added accessory protein (Fig. 1A). This is consistent with the stoichiometric action that has been previously demonstrated for hog \( \tau \) (Weingarten et al., 1975; Wittman et al., 1976; Cleveland et al., 1977b; Herzog and Weber, 1978b), for hog HMW protein (Herzog and Weber, 1978b), and for bovine HMW protein (Kim et al., 1979). A qualitatively similar set of profiles of assembly are shown in Fig. 1B for a parallel experiment using crude hog accessory proteins, although the specific activity of the crude hog associated proteins appears to be about 25% higher. In these parallel experiments, assembly reached only 50 to 60%, which could reflect some denaturation of the tubulin or, alternatively, a systematic error in the concentration of bushy stunt virus used in the quantitative electron microscopy.

Using the quantitative electron microscopy assay for ability of the chicken accessory fraction to stimulate assembly of hog tubulin, we have purified a chicken \( \tau \) activity by the procedure used to purify hog \( \tau \) (Cleveland et al., 1977b). Fig. 2 shows a series of assays at varying associated protein concentrations for three steps (phosphocellulose, ammonium sulfate, and hydroxyapatite chromatography) in the purification of chicken brain \( \tau \). Specific microtubule-inducing activity (defined as the ratio of the concentration of crude \( \tau \) necessary to induce 50% polymerization of available tubulin/the corresponding concentration of the fraction being assayed) increases 4-fold through the purification procedure. This is a similar degree of purification as previously reported for hog \( \tau \) (Cleveland et al., 1977b; Herzog and Weber, 1978b). Total microtubule-inducing activity present in the purified \( \tau \) fraction contains 30% of the initial stimulatory activity recoverable...
of aromatic amino acids. Amino acid analysis of purified chicken brain \( \tau \) was performed on both material purified on hydroxypatite and on material subsequently purified by gel electrophoresis (Table 1, Columns 1 and 2). The results of that analysis are in close agreement with that previously reported for hog \( \tau \) (Column 3). Proline is seen to be prominent in both \( \tau \)s, as are glycine, glutamic acid, serine, and lysine. Both \( \tau \)s are poor in methionine and in aromatic amino acids.

**Peptide Mapping of Chicken and Hog \( \tau \) and Chicken and Hog HMW Protein**—Although the hog and chicken \( \tau \)s were purified by analogous protocols, were microheterogeneous, had similar molecular weights, had nearly identical amino acid compositions, and shared common physical characteristics, we examined further the possible relationships among the different chicken \( \tau \) bands and their hog \( \tau \) counterparts by one-dimensional peptide mapping. Individual protein bands were isolated from initial polyacrylamide gels and aliquots were rerun on a second low per cent acrylamide gel to verify their purity (Fig. 5). The remaining portions of excised bands were proteolyzed by addition of chymotrypsin and the peptide fragments were resolved on a 15% polyacrylamide gel. Results for the hog and chicken \( \tau \) bands are shown in Fig. 6. The four

![Fig. 7. SDS-polyacrylamide gel of chicken \( \tau \) present in associated protein fractions obtained from different protocols of purification. Lane 1, chicken associated proteins prepared from the two-cycle microtubule protein prepared by the protocol of Dentler et al. (1974) which elute from phosphocellulose at 0.29 M KCl; Lane 2, the heat-stable non-tubulin proteins obtained by boiling our two-cycle microtubule protein according to the protocol of Fellous et al. (1977) as modified by Herzog and Weber (1978); Lane 3, our normal chicken \( \tau \) purified through ammonium sulfate fractionation; Lane 4, purified hog \( \tau \) (Cleveland et al., 1977b).](image)

from phosphocellulose. An SDS-polyacrylamide gel of the corresponding fractions is shown in Fig. 3. A group of closely spaced bands of molecular weights between 53,000 and 62,000 is enriched by the purification procedure. This group of proteins comprises about 15 to 20% of the associated proteins. Purified chicken \( \tau \) is shown in Fig. 3, Lane 11. While none of these chicken \( \tau \) polypeptides precisely co-electrophoreses with any hog \( \tau \) band (see Fig. 7, Lanes 3 and 4), the pattern of closely spaced bands of approximately \( M_r = 60,000 \) is quite similar to that found for hog \( \tau \).

**Properties of Chicken Brain \( \tau \)**—For further characterization of the chicken brain \( \tau \) activity, a sample of \( \tau \) purified through ammonium sulfate fractionation was centrifuged through a 5 to 25% sucrose gradient. Fractions were collected from the gradient, desalted into Buffer P, and assayed for ability to stimulate tubulin polymerization. The profile of optical density at 280 nm and assays for inducing polymerization of tubulin are shown in Fig. 4. A single peak of activity, sedimenting with an \( s_{20w} \) of approximately 2.8 S can be observed, in agreement with the 2.7 S value reported in a similar experiment for the activity of hog \( \tau \) (Penningroth et al., 1976). An SDS-polyacrylamide gel of fractions from the gradient (not shown) indicated that the \( \tau \) polypeptides sedimented with the peak of microtubule stimulatory activity and that the shoulder on the optical density profile corresponded to higher molecular weight contaminants.

Purified chicken brain \( \tau \) has an \( E_{1%}^\text{280} \) of 2.9, which is very similar to the value of 2.8 measured previously for hog brain \( \tau \) (Cleveland et al., 1977c; Herzog and Weber, 1978b). This is an unusually low extinction coefficient reflecting the low levels

![Fig. 8. Purification of \( 35^\text{S} \)-labeled SV3T3 microtubule proteins by co-polymerization with porcine brain microtubules. Labeled SV3T3 microtubule proteins were isolated from cell extracts by co-polymerization with porcine brain microtubule protein, followed by phosphocellulose chromatography as described under "Materials and Methods." Aliquots of the mixture were taken from each cycle of sedimentation and were quickly frozen. They were later thawed, precipitated with trichloroacetic acid, and centrifuged, and the precipitated protein was analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. The following standards were run in parallel and their positions are marked to the right of the figure: HMW, porcine brain high molecular weight protein; \( \text{TAU} \), porcine brain \( \tau \); \( \tau \), porcine brain tubulin; \( \text{P} \), rabbit muscle actin. Also marked is the position of a \( M_r = 50,000 \) protein which is a prominent copolymerization product. A, crude extract; B, 1st cycle of polymerization; C, second cycle; D, third cycle; E, third cycle material not adsorbed to phosphocellulose; F, 1.0 M NaCl wash from phosphocellulose.](image)
chicken τ bands were analyzed independently. The hog τ bands were analyzed in two fractions, one comprising the two higher molecular weight hog polypeptides and another comprising the two lower molecular weight polypeptides. Previous peptide mappings have shown that the individual bands in the higher molecular weight doublet are virtually identical and similarly the bands in the lower molecular weight doublet are nearly identical (Cleveland et al., 1977c). The two doublets are also closely related to each other. Each analyzed band has been displayed in three slots to which have been added increasing amounts of chymotrypsin. Shown below the fluorogram of the peptide patterns in Fig. 6 is a schematic drawing of the third slot of each triplet of the peptide patterns. The length of the line representing a band in the schematic drawing indicates the approximate relative intensity of that band in the pattern. Prominent bands have been numbered for ease of reference, with Band 0 representing the undigested protein. It is evident in Fig. 6, E to H, that, although several differences are readily discernible among the patterns of the four chicken τ bands (for example, peptide 5 is present only in the two higher molecular weight chicken τ polypeptides, Fig. 6, E and F), the overall appearance of the four patterns is strikingly similar. Moreover, the chicken τ patterns are qualitatively very similar to the compared hog τ polypeptides, although the lowest chicken peptide fragments (Lines 9 to 12 in the schematic drawing, Fig. 6) run at slightly lower apparent molecular weights than the corresponding hog fragments. Such similarity of peptide patterns suggests that 1) the chicken τ polypeptides represent a family of closely related species and 2) the chicken brain τ polypeptides are also very similar to their hog brain τ counterparts.

A similar experiment using the major hog and chicken HMW bands (i.e. MAP 2 of Rosenbaum and co-workers; for example, see Sloboda et al., 1976) isolated from an initial gel is shown in Fig. 6, A and B. Major similarities between the chicken and hog HMW protein patterns cannot be recognized.

**Chicken τ May Be Isolated from Microtubules Purified by Other Protocols**—In an attempt to reconcile conflicting reports on the presence of τ in microtubules, we have prepared chicken accessory proteins by three other procedures. First, microtubule protein prepared by the temperature-dependent, glycerol-free assembly procedure (in Pipes buffer, pH 6.9) used by Rosenbaum and co-workers (Dentler et al., 1974; Sloboda and Rosenbaum, 1979) was taken through three cycles of temperature-dependent assembly, and tubulin was separated from accessory protein fractions by chromatography on phosphocellulose (using the Sloboda et al. (1976) modifications of the Weingarten et al. (1975) protocol). Adsorbed accessory proteins were then eluted with a linear KCl gradient instead of the stepwise elution used in the procedure of Sloboda and Rosenbaum (1979). A major protein species co-electrophoresing with purified chicken τ elutes at 0.29 M KCl (Fig. 7, Lane 1), similar to the elution of chicken τ at 0.27 M NaCl in Mes buffer, as reported above, and the elution at 0.25 M NaCl of hog brain τ by our laboratory (Weingarten et al., 1975; Penninger et al., 1976; Cleveland et al., 1977b) and by Herzog and Weber (1978b). Second, using microtubule protein prepared both by our usual temperature-dependent assembly procedure (Weingarten et al., 1975) as well as by the glycerol-free method of Dentler et al. (1974), heat-stable microtubule accessory proteins were prepared by the Herzog and Weber (1978b) modification of the Fellous et al. (1977) protocol. Shown in Lane 2 of Fig. 7 are the heat-stable proteins prepared from chicken microtubule proteins obtained in Fig. 6. E to H, that, although several differences are readily discernible among the patterns of the four chicken τ bands (for example, peptide 5 is present only in the two higher molecular weight chicken τ polypeptides, Fig. 6, E and F), the overall appearance of the four patterns is strikingly similar. Moreover, the chicken τ patterns are qualitatively very similar to the compared hog τ polypeptides, although the lowest chicken peptide fragments (Lines 9 to 12 in the schematic drawing, Fig. 6) run at slightly lower apparent molecular weights than the corresponding hog fragments. Such similarity of peptide patterns suggests that 1) the chicken τ polypeptides represent a family of closely related species and 2) the chicken brain τ polypeptides are also very similar to their hog brain τ counterparts.

A similar experiment using the major hog and chicken HMW bands (i.e. MAP 2 of Rosenbaum and co-workers; for example, see Sloboda et al., 1976) isolated from an initial gel is shown in Fig. 6, A and B. Major similarities between the chicken and hog HMW protein patterns cannot be recognized.

**Chicken τ May Be Isolated from Microtubules Purified by Other Protocols**—In an attempt to reconcile conflicting reports on the presence of τ in microtubules, we have prepared chicken accessory proteins by three other procedures. First, microtubule protein prepared by the temperature-dependent, glycerol-free assembly procedure (in Pipes buffer, pH 6.9) used by Rosenbaum and co-workers (Dentler et al., 1974; Sloboda and Rosenbaum, 1979) was taken through three cycles of temperature-dependent assembly, and tubulin was separated from accessory protein fractions by chromatography on phosphocellulose (using the Sloboda et al. (1976) modifications of the Weingarten et al. (1975) protocol). Adsorbed accessory proteins were then eluted with a linear KCl gradient instead of the stepwise elution used in the procedure of Sloboda and Rosenbaum (1979). A major protein species co-electrophoresing with purified chicken τ elutes at 0.29 M KCl (Fig. 7, Lane 1), similar to the elution of chicken τ at 0.27 M NaCl in Mes buffer, as reported above, and the elution at 0.25 M NaCl of hog brain τ by our laboratory (Weingarten et al., 1975; Penninger et al., 1976; Cleveland et al., 1977b) and by Herzog and Weber (1978b). Second, using microtubule protein prepared both by our usual temperature-dependent assembly procedure (Weingarten et al., 1975) as well as by the glycerol-free method of Dentler et al. (1974), heat-stable microtubule accessory proteins were prepared by the Herzog and Weber (1978b) modification of the Fellous et al. (1977) protocol. Shown in Lane 2 of Fig. 7 are the heat-stable proteins prepared from chicken microtubule proteins obtained in Fig. 6.

**Fig. 9. Peptide maps of τ from SV3T3 cells.** Total microtubule associated proteins from SV3T3 cells were prepared with 1.0 M NaCl wash of phosphocellulose to which had been applied SV3T3 microtubule protein obtained from three cycles of co-polymerization as in Fig. 8. τ bands from the SV3T3 cells were separated from other associated proteins by electrophoresis on an 8.5% SDS-polyacrylamide gel. Excision of the SV3T3 τ bands utilized the positions of the cold τ bands (visible after brief staining) as a guide. Proteins from excised slices were eluted by diffusion and precipitated by addition of trichloroacetic acid to 20% final concentration and 100 μg/ml of unlabeled tubulin. After the extraction, the proteins were resuspended in 50 μl of digestion buffer and proteolyzed with 4 μg of chymotrypsin. Samples were electrophoresed on 15% gels and were subjected to fluorography. A to C, excised bands rerun on an 8.5% gel to verify their purity: A, 14C-labeled hydroxyapatite-purified hog τ; B, SV3T3 τ band co-electrophoresing with the M, = 62,000 hog τ band; C, SV3T3 τ band co-electrophoresing with the M, = 62,000 hog τ band. D to F, peptide maps from the 15% gel: D, whole hog τ as in A; E, the lower molecular weight SV3T3 τ band; F, the higher molecular weight 3T3 τ band. Shown at the bottom is a schematic drawing of the digestion products of (D to F). Numbering of digestion products is as in Fig. 6.
using the Weingarten et al. (1974) protocol. Similar results are obtained by heat precipitation of microtubule protein prepared by the method of Dentler et al. (1974) (not shown). An aliquot of purified chicken N bands is shown in Lane 3 (Fig. 7). Chicken N bands are present in all these preparations of associated proteins. Also present is a prominent high molecular weight species. This result for chicken brain is, therefore, in agreement with Herzog and Weber (1973b) for hog brain, who showed that both N and HMW proteins are present in microtubule preparations and that both are heat-stable.

**Purification of SV3T3 Microtubule Proteins by Co-polymerization with Purified Microtubules**—Recent immunofluorescence experiments of Connolly et al. (1977) suggested that mouse fibroblasts contain an antigen related to hog brain N in their interphase and mitotic microtubule arrays. To test whether chemically identifiable N protein could be found in microtubules from mouse fibroblasts, we purified microtubules from an SV40-transformed mouse 3T3 cell line (SV3T3) by co-polymerization of [35S]methionine-labeled protein with unlabeled carrier hog brain microtubules using the procedure of Spiegelman et al. (1977). After three cycles of co-assembly (as described under “Materials and Methods”), the SV3T3 microtubule proteins were fractionated on phosphocellulose and aliquots were applied to an SDS-polyacrylamide gel (Fig. 8). Tubulin is barely visible in the total soluble proteins (Fig. 8A). After three cycles of co-polymerization, the labeled proteins are composed of about 85% tubulin (Fig. 8D). The material from the third cycle then was fractionated on phosphocellulose into two fractions, one composed of nearly radiologically homogeneous tubulin (Fig. 8E) and the other composed of a series of associated proteins (Fig. 8F). These total SV3T3 microtubule-associated proteins are comprised of a large spectrum of proteins of high and low molecular weights. Most prominent are the two very high molecular weight proteins and a Mr = 50,000 band. Also visible are two bands at 56,000 and 62,000 which precisely co-electrophoresed with two of the carrier hog N bands. To determine whether these bands represent N proteins from mouse cells, the bands were excised from a gel after brief staining and peptide-mapped along with hog brain N. Chymotryptic digestion patterns of hog brain N and of the co-electrophoresing SV3T3 polypeptides are displayed on a 16% gel in Fig. 9. A schematic drawing of the gel patterns is shown at the bottom of the figure.

There are many common peptides among whole hog brain N and the 56,000 and 62,000 SV3T3 associated protein bands (e.g. 4 to 8 in the numbering sequence of Fig. 6). Thus, it is clear that mouse associated proteins purified by cycles of co-polymerization contain protein species which co-electrophoresed with hog brain N polypeptides and which yield peptide patterns virtually identical to those of hog brain N.

A similar peptide mapping experiment was performed using hog brain HMW protein (MAP 2) and its co-electrophoresing SV3T3 counterpart (Fig. 10). It is evident that, like the results for N, in Fig. 9, the hog and mouse high molecular weight proteins are virtually indistinguishable in these one-dimensional fingerprints.

**DISCUSSION**

The total and relative amounts of the individual associated proteins which co-purify with tubulin through cycles of assembly vary as a function of the conditions of the polymerization procedure (Murphy and Borisy, 1975; Sloboda et al., 1976; Keates and Hall, 1975; Scheele and Borisy, 1976; Cleveland et al., 1977b). Microtubule protein from hog brain purified by the method of Shelanski et al. (1973) (as modified by Weingarten et al. (1974)) consists of 90% tubulin, with the remaining 10% distributed among non-tubulin proteins of both high and low molecular weights. Moreover, a majority of the activity for stimulating assembly of tubulin is assayed in Mes buffer, pH 6.4, is present in a class of proteins called N protein, which represents a series of closely related polypeptides of molecular weights between 55,000 and 62,000 (Cleveland et al., 1977b). In addition, a considerable amount of activity is found in fractions containing very high molecular weight peptides (Cleveland et al., 1977b). Borisy and co-workers (Murphy and Borisy, 1975; Borisy et al., 1975) have reported that preparations of hog microtubules purified in Pipes buffer in the absence of glycerol consist of 75% tubulin, 15% high molecular weight components (greater than 250,000), and 10% in other non-tubulin components. These investigators have reported that all associated protein fractions (high or low molecular weight) are about equally effective in stimulating assembly when compared on a weight basis.

The large differences in the associated proteins obtained from different protocols, together with the apparent ability of various polycationic substances to promote microtubule or microtubule-like assembly from tubulin (Erickson, 1976; Erickson and Voter, 1976), initially cast some doubt on the specificity or importance of the tubulin/associated protein interaction. Although several laboratories (Borisy et al., 1975; Sloboda et al., 1976; Berkowitz et al., 1977) demonstrated the constant stoichiometry of tubulin and of some accessory proteins throughout multiple cycles of assembly, Lee et al. (1978) showed clearly that this is an insufficient criterion for specificity. In addition, tubulin will assemble in the absence of detectable non-tubulin proteins in phosphate buffer contain-
ing high concentrations of Mg$^{2+}$ and glycerol (Lee and Timasheff, 1975). Pipette buffer containing dimethyl sulfoxide (Himes et al., 1976), high concentrations of magnesium (Herzog and Weber, 1977), dextran (Herzog and Weber, 1978a), and polyethylene glycol (Hersg and Weber, 1978a). Nonetheless, recent reports using indirect immunofluorescence have clearly documented the presence of proteins antigenically related to porcine $\tau$ (Connolly et al., 1977) and to porcine high molecular weight protein (Connolly et al., 1977) in spindles and in intracellular microtubule arrays of secondary mouse fibroblasts and rat glial cells, respectively. Using an antibody raised against rat HMW protein, Sherline and Schiavone (1977, 1978) have reported similar staining of microtubule networks in 3T3 cells and neuroblastoma cells and staining of mitotic spindles in 3T3 cells. Moreover, Herzog and Weber (1978b) have resolved some apparent contradictions in previous work on associated proteins by demonstrating the purification of active fractions of $\tau$ and HMW protein from the same preparation of microtubules.

In the present report we have purified $\tau$ from chicken brain microtubules. The $\tau$ fraction comprises over 30% of microtubule-stimulating activity recoverable from phosphocellulose. The purification is quite analogous to that previously reported for hog brain $\tau$ (Cleveland et al., 1977b) and yields a set of closely spaced bands of molecular weights between 53,000 and 62,000. While none of the chicken polypeptides precisely co-electrophoreses with any of the hog $\tau$ bands, the one-dimensional peptide maps given in Fig. 6 demonstrate striking similarities among the individual chicken bands and, moreover, between corresponding chicken and hog polypeptides. The hog and chicken $\tau$s have been found to possess similar sedimentation coefficients (2.7 and 2.8 S, respectively) and similar amino acid compositions (Table I). Both $\tau$s have low $E_{280}$ values of approximately 2.8. Both are heat-stable. These results clearly demonstrate 1) the presence of a $\tau$ species capable of stimulating tubulin assembly in vitro in chicken brain microtubules and 2) the conserved nature of the physical properties and peptide fingerprints of the hog and chicken proteins.

Although $\tau$ has been identified and purified from hog brain (Penningroth et al., 1976; Cleveland et al., 1977b; Murphy et al., 1977; Herzog and Weber, 1978b) and from rat brain (Follows et al., 1977), a recent report suggested that $\tau$ is either absent or inactive in chicken brain (Sloboda and Rosenbaum, 1979). The apparent discrepancy with the present results arises from a misidentification of the $\tau$ peptides. Sloboda and Rosenbaum (1979) seemed to have assumed that chicken $\tau$ should elute at 0.18 M KCl because this fraction contained some proteins of similar molecular weight to hog brain $\tau$. The authentic chicken $\tau$ polypeptides, as demonstrated above, elute at 0.29 M KCl and not at 0.18 M KCl and, therefore, an identification of two inactive 60,000-dalton polypeptides which elute at 0.18 KCl as $\tau$ protein is certainly in error.

Tubulin and associated proteins have recently been prepared in a number of laboratories by direct polymerization of microtubules from nonneuronal cells. Theoretically, direct polymerization has both advantages and disadvantages as compared to co-polymerization. For example, in direct polymerization there are no carrier associated proteins which might compete with the endogenous nonneuronal associated proteins and lead to a decrease in their representation. A disadvantage, however, of direct polymerization is that initial assembly takes place in a very concentrated cell extract. This may increase the opportunities for nonspecific interaction of cellular components. Therefore, it is difficult to say a priori which procedure will most accurately reflect the composition in vivo.

Wiche and Cole (1976) first reported the successful direct polymerization of tubulin from cultured cells, using a rat glial cell line (C6). They used the protocol of Shelanski et al. (1973) which included 4 M glycerol to promote assembly. These authors reported that, following two cycles of assembly, microtubule preparations from C6 cells were composed of 95% of tubulin with the remaining 5% consisting predominantly of actin, a polypeptide of around $M_r = 50,000$, and a brain-like high molecular weight associated protein. Later Wiche et al. (1977) reported polymerization of microtubules from 3T3 and SV3T3 cells and again found the microtubule protein to consist of 95% tubulin, although no data was presented on the nature of the remaining 5% non-tubulin proteins. Weber et al. (1977) also purified microtubules directly from SV3T3 cells using cycles of glycerol-stimulated assembly. Tubulin purity again reached the 95% level, with actin representing the major non-tubulin protein. Additional non-tubulin proteins were not characterized. Although Nagle et al. (1977) reported polymerization in the presence of glycerol from a variety of cell types, only with neuroblastoma cells was assembly taken through more than one cycle. In this later experiment, a large number of non-tubulin proteins were identifiable, including a 49,000-dalton peptide and a number of peptides between 52,000 and 68,000. Polypeptides of molecular weights near those of the brain high molecular weight associated proteins, however, were not seen. Weatherbee et al. (1978) prepared microtubules from HeLa cells with and without glycerol to stimulate polymerization. A $M_r = 68,000$ non-tubulin protein was found by these authors to co-purify with microtubules; polypeptides corresponding to brain high molecular weight components were not observed. Finally, Bulinski and Borisy (1979) have also reported the direct assembly of tubulin from HeLa cells using a protocol without glycerol. Following two cycles of assembly, 95% of the protein corresponded to tubulin. No proteins co-electrophoresing with brain $\tau$ or HMW protein were observed. Prominent HeLa cell associated proteins were identified as a triplet just above $M_r = 200,000$ and as a doublet near $M_r = 120,000$. A $M_r = 68,000$ component, while detectable in two-cycle material, was not present in succeeding cycles.

There is, therefore, considerable disparity in the reported non-tubulin accessory proteins purified with microtubules which have been directly polymerized from cultured cells. Although each laboratory has reported tubulin purity to approach 95%, qualitatively different accessory protein profiles have been obtained even when the same cell types were used. This may reflect the nonspecificity of many such interactions. The potential for artifact in polymerization procedures has recently been discussed by Lee et al. (1978). However, at the very least polymerization or co-polymerization is useful for purifying previously identified microtubule associated proteins, as has been done in the present report.

Using co-polymerization rather than direct polymerization, we have identified several SV3T3 cell proteins which co-purify with microtubules. Like Wiche and Cole (1976) and Nagle et al. (1977), we have found a $M_r = 50,000$ protein to co-purify through three cycles of assembly, along with a small amount of actin. In addition, we find a number of other polypeptides of both high and low molecular weights. Among these latter components, a high molecular weight component (MAP 2) and $\tau$ have been clearly identified by electrophoretic mobility and peptide fingerprinting. This finding was not unexpected in view of the immunofluorescent results (see above) of Connolly et al. (1977, 1978) and of Sherline and Schiavone (1977, 1978). On the other hand, similar experiments with Chinese hamster ovary cells and neuroblastoma cells have been performed in this laboratory (Spiegelman, 1978) in which accessory protein bands which co-electrophoresed with carrier $\tau$
and high molecular weight proteins could not be easily identified.

Whether all cell types contain brainlike $\tau$ or HMW species as do mouse fibroblasts remains, then, an open question. What is clear, however, is the presence together of both $\tau$ and HMW protein in microtubules of a single nonneuronal cell type. This, when combined with the similarity of the $\tau$ proteins from hog brain and chicken brain in many physical and chemical properties, suggests that $\tau$ protein is highly conserved and probably widely distributed. Although less data has been presented here, the similarity of the peptide maps of the mouse fibroblast HMW protein and hog brain HMW protein suggests that this protein may also be widely distributed and conserved.

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REFERENCES