Two tubulin variants, isolated from chicken brain and erythrocytes and known to have different peptide maps and electrophoretic properties, are demonstrated to exhibit different assembly properties in vitro: 1) erythrocyte tubulin assembles with greater efficiency (lower critical concentration, greater elongation rate) but exhibits a lower nucleation rate than brain tubulin, and 2) erythrocyte tubulin readily forms oligomers whose presence significantly retards the rate of elongation, suggesting that tubulin oligomers may also be important for determining the rate of assembly and the length of microtubules in erythrocytes. Erythrocyte tubulin isolated by cycles of in vitro assembly-disassembly is also demonstrated to contain a 67-kDa tau factor that greatly enhances microtubule nucleation but has little effect on elongation rates or critical concentration. Immunofluorescence microscopy with tau antibody indicates that tau is specifically associated with marginal band microtubules, suggesting that it may be important for determining microtubule function in vivo.

In this paper we compare the mechanisms of microtubule self-assembly (nucleation and elongation) for two preparations of microtubule protein in the chicken, one from brain tissue, the other from erythrocytes from the blood. These preparations exhibit major differences in their self-assembly in vitro, and have been shown to differ from each other in the kinds of MAPs and tubulin isoforms that they contain (1, 2). Our goal here is to establish the relative significance of tubulin isoforms for self-assembly in vitro, since the information will be useful for understanding the control of microtubule assembly in the cell.

As isolated by cycles of an in vitro assembly procedure, brain microtubules contain both high molecular weight MAPs (high M, MAPs) (3, 4) and intermediate molecular weight MAPs called tau (5, 6), whereas erythrocyte microtubules contain only the intermediate molecular weight factors similar to tau (2). Sloboda and Dickersin (7) reported that MAP-2 is associated with marginal band microtubules in vertebrate erythrocytes, but so far we have not been able to confirm this observation. Both classes of MAPs are known to contain assembly-promoting activity and co-purify with microtubules through cycles of in vitro purification (4, 5, 8-10). Preliminary studies on the mechanism by which high M, MAPs stimulate assembly have already been reported (9, 11), but the details regarding tau-promoted assembly remain unclear. In this paper, we establish that the 50-70-kDa factors in erythrocyte microtubule preparations are tau and that their primary effect is to stimulate microtubule assembly by promoting microtubule nucleation.

Another approach to the problem of understanding the regulation of tubulin assembly and function has been to look for naturally occurring tubulin variants (isoforms), since differences in the dynamics of microtubule activity may be due to biochemical differences in the tubulin subunits themselves (12). Indeed, tubulin isoforms generated through post-translational modification (13, 14) and by differential gene expression (15, 16) have been shown to be related to specific programs of cell and tissue development (17-22). In most cases, however, it has not yet been possible to describe the molecular detail how the properties of microtubule assembly and function are related to the chemical attributes of a specific variant. Recently we described the occurrence of two tubulin variants in the erythrocytes and brain tissue of the chicken (1). We have shown that the variants contain biochemically distinct β-tubulin subunits, which on the basis of peptide mapping and amino acid composition, appear to be different gene products. The erythrocyte β-tubulin subunit is relatively more alkaline and hydrophobic than the β subunits in brain and other tissues. Compared with brain tubulin, erythrocyte tubulin exhibits a slower assembly rate and a lower critical concentration (0.1 mg/ml) and produces long, stable polymers similar to those contained in the marginal bands of erythrocytes (23).

In this paper we present evidence that the major differences in assembly of brain and erythrocyte microtubules are partly due to the tubulin subunits themselves, establishing for the first time that different tubulin variants can exhibit different assembly properties in vitro. Finally, we show through in vitro studies of erythrocyte tubulin assembly that the rate of microtubule elongation is significantly retarded in subunit preparations containing tubulin oligomers, and we speculate on the significance of tubulin oligomers in regulating microtubule assembly in vivo.

MATERIALS AND METHODS

Isolation of Microtubule Protein, Tubulin, and Tau—Microtubule protein was isolated by two cycles of in vitro assembly and disassembly.

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Erythrocyte Tubulin Assembly

bly from chicken brain by the procedure of Dentler et al. (3) and from chicken erythrocytes by a procedure described by us in a previous report (2). Tubulin from both sources was purified (>99%) from microtubule protein by ion exchange chromatography on phosphocellulose P-11 (Whatman) by the procedure described by Weigarten et al. (5). The purified tubulin is designated PC-tubulin. 1 For the preparation of tubulin, non-tubulin proteins (bound fraction) were eluted from phosphocellulose by elution with a NaCl gradient (0.1-0.0 M NaCl in 25 mM Pipes, pH 6.6). The peak tau fractions were pooled, dialyzed against assembly buffer minus GTP, and stored at -90°C.

Measurement of Microtubule Self-assembly and Microtubule Elongation—Unless otherwise specified, the microtubule assembly buffer for all preparations was 0.1 M Na/Pipes, pH 6.94, containing 1.0 mM MgCl₂, 1.0 mM GTP, and 5% glycerol.

Self-assembly was monitored by recording the change in optical density at 350 nm in a Gilford spectrophotometer equipped with a water-jacketed cuvette holder adjusted to 37°C.

Microtubule elongation (subunit addition onto the ends of exogenous, preformed microtubules) was performed using the guidelines established previously by Johnson and Borisy (11, 24). Typically, a 0.8-ml aliquot of subunits at 0°C was placed in a cuvette and warmed to polymerization temperature (30°C). The temperature increased exponentially, with the time required for half-maximal equilibration being less than 5 s. Tubulin subunits (microtubule protein and PC-tubulin) failed to polymerize during the period of measurement with and without the addition of microtubule seeds. Microtubule seeds were prepared by mechanically shearing polymerized microtubules (brain microtubule protein, 5 mg/ml) by passage through a 22-gauge syringe needle and were not used after 2 h. Examination of microtubule seeds by electron microscopy revealed no redistribution in seed length for up to 5 h. The constancy of microtubule number concentration was also examined independently by examining the linearity of plots of initial rate of elongation vs. seed length for up to 5 h. The constancy of microtubule number concentration was also examined independently by examining the linearity of plots of initial rate of elongation vs. seed length for up to 5 h. The constancy of microtubule number concentration was also examined independently by examining the linearity of plots of initial rate of elongation vs. seed length for up to 5 h. The constancy of microtubule number concentration was also examined independently by examining the linearity of plots of initial rate of elongation vs. seed length for up to 5 h. The constancy of microtubule number concentration was also examined independently by examining the linearity of plots of initial rate of elongation vs. seed length for up to 5 h. The constancy of microtubule number concentration was also examined independently by examining the linearity of plots of initial rate of elongation vs. seed length for up to 5 h. The constancy of microtubule number concentration was also examined independently by examining the linearity of plots of initial rate of elongation vs. seed length for up to 5 h. The constancy of microtubule number concentration was also examined independently by examining the linearity of plots of initial rate of elongation vs. seed length for up to 5 h. The constancy of microtubule number concentration was also examined independently by examining the linearity of plots of initial rate of elongation vs. seed length for up to 5 h. The constancy of microtubule number concentration was also examined independently by examining the linearity of plots of initial rate of elongation vs. seed length for up to 5 h. The constancy of microtubule number concentration was also examined independently by examining the linearity of plots of initial rate of elongation vs. seed length for up to 5 h. The constancy of microtubule number concentration was also examined independently by examining the linearity of plots of initial rate of elongation vs. seed length for up to 5 h. The constancy of microtubule number concentration was also examined independently by examining the linearity of plots of initial rate of elongation vs. seed length for up to 5 h. The constancy of microtubule number concentration was also examined independently by examining the linearity of plots of initial rate of elongation vs. seed length for up to 5 h.

We established that the criteria established by Johnson and Borisy (11, 24) were met by our protocol for measuring initial rates: 1) no microtubules were formed in the absence of seeds (confirmed by electron microscopy); 2) the initial rate of assembly was directly proportional to the number concentration of seeds added (determined by mechanical shearing); and 3) the change in optical density following the addition of seeds exhibited a single pseudo-first-order exponential, indicating that the reaction proceeded toward completion with no change in the microtubule number concentration and therefore no initiation (determined by visual inspection of the spectrophotometer tracings). These criteria were generally evaluated on each day of an experiment to guarantee consistency in results and interpretations.

SDS-Polyacrylamide Gel Electrophoresis—SDS-slab gels containing 12% acrylamide were prepared by the method of Laemmli (25) except that 0.4% diallyl tartardiamide was used as the cross-linker (26). Acrylamide and cross-linker were from Bio-Rad. SDS (99% as sodium dodecyl sulfate) was obtained from British Drug House. Gels were stained with Coomassie Blue and destained as described by Fairbanks et al. (27).

Immunoblotting—Tau polypeptides were identified by examining the binding of two affinity-purified rabbit antibodies directed against mammalian tau and to chicken erythrocyte tau to components in gels and blotted by electrophoretic transfer onto nitrocellulose by the method of Towbin et al. (28). The procedures for processing the nitrocellulose sheets for autoradiography after secondary labeling with ³²P-labeled protein A have been described elsewhere (29).

Identification of Tau as a Microtubule-associated Protein in Erythrocytes

Erythrocyte microtubules prepared by two cycles of in vitro assembly contain 95% tubulin, 4% hemoglobin, plus other trace components, of which about half (i.e. 0.5% of the total protein) consists of a 67-kDa polypeptide similar in size to tau, a microtubule-associated protein with assembly-promoting activity (Fig. 2).

The following observations established the identity of the 67-kDa factor as tau. 1) Erythrocyte tau remained soluble after heat treatment (5) and eluted on agarose columns with a similar distribution coefficient as brain tau (Kₐ = 0.7 on Bio-Gel A-1.5m) (31). 2) As determined by immunoblotting, a rabbit antibody to hog brain tau (generous gift from the lab of Marc Kirschner, University of California, San Francisco) cross-reacts with tau components in chicken brain and chicken erythrocytes, including the erythrocyte 67-kDa com-

Biochemical Materials—Pipes, sodium salt, was obtained from Calbiochem Behring; all other chemicals were obtained from Sigma.

RESULTS

Microtubule protein from erythrocytes polymerizes slowly compared to preparations from brain tissue. Erythrocyte microtubule assembly in vitro typically exhibits a long lag time prior to the onset of assembly and a slower maximum rate of polymerization (Fig. 1). However, the critical concentration for erythrocyte microtubule assembly (0.05-0.10 mg/ml), although variable between preparations, is usually lower than that for brain. The combination of low critical concentration and slow rate of assembly is a characteristic feature of erythrocyte tubulin assembly. The inset to Fig. 1 shows that microtubule growth occurs immediately upon addition of microtubule seeds to prewarmed erythrocyte tubulin subunits during the lag period. Thus, subunit activation or inhibitor repression does not appear to be required for the addition of tubulin subunits on the ends of microtubule seeds. In this paper we compare preparations of microtubule protein from chicken brain and erythrocytes with respect to their properties of nucleation, growth, and critical concentration to determine to what extent the differences in microtubule self-assembly are due to differences in the tubulin variants and to MAPs.

![Fig. 1. Self-assembly of microtubule protein from chicken brain and erythrocytes. The assembly buffer is 0.1 M Pipes, 1 mM Mg/GTP, and 5% glycerol at pH 6.8. Erythrocyte protein exhibits a longer lag time and a slower assembly rate than brain protein. Inset, addition of erythrocyte tubulin subunits on exogenous microtubule seeds. Microtubule protein (2.0 mg/ml) in assembly buffer was prewarmed for 50 s at 37°C and mixed with an equal volume of seeded erythrocyte seeds at the same concentration (A). A control aliquot received no further additions (B). The final protein concentration in both cuvettes was 2.0 mg/ml.](image-url)
component (Fig. 2). In addition, our own rabbit antibody to the erythrocyte 67-kDa component recognizes tau in chicken brain and erythrocytes but does not bind to tau from hog brain (Fig. 2). Based on these criteria, we refer to the 67-kDa protein as erythrocyte tau. In addition to the 67-kDa species, other trace components were present which specifically bound to antibody and therefore are assumed to be tau isoforms. The molecular weights of these components as determined by SDS-polyacrylamide gel electrophoresis were 75,000, 70,000, 61,000, 56,000, and 52,000. In chicken brain a 61-kDa component was observed to be the major, and the 67-, 56-, 54-, and 52-kDa components the minor, tau species. The possibility that these isoforms may represent different degrees of phosphorylation of the same tau polypeptide (6, 32) has not been examined.

To determine if the 67-kDa protein also stimulated assembly of microtubules, a nontubulin fraction eluted from a phosphocellulose column with 1.0 M KCl was further fractionated by gel filtration chromatography on an agarose A-1.5m column. Fractions were mixed with PC-tubulin at 37 °C and monitored by spectrophotometry at 350 nm to determine which proteins contained assembly-promoting activity (Fig. 3). Although the total nontubulin fraction contained many components, each protein had a unique elution position on the column. A single peak of assembly-promoting activity was coincident with the elution position of the 67-kDa component and other minor tau isoforms. One major high molecular weight component and several minor components (<50 kDa) did not appear to contain stimulatory activity.

Erythrocyte Tau Binds to Microtubules and Nucleates Tubulin Assembly

PC-tubulin self-assembles poorly by itself (Fig. 4A, tracing = 0% tau) but polymerizes more rapidly and with shorter lag times with increasing amounts of the nontubulin fraction. Nearly identical results were obtained when polymerization was measured by turbidity or by viscometry; in both instances the rate of assembly increased linearly while the extent of polymerization remained nearly constant over a broad range of concentrations of the nontubulin fraction (Fig. 4B). Although comparison of fractions lacking and containing tau indicates a difference in the extent of polymer formation and hence a difference in the critical concentration, this is actually not the case. After prolonged incubation (60 min), samples lacking tau were observed to contain microtubules and to attain the same equilibrium level of turbidity as samples containing tau. Thus, although tau does not appear to shift the critical concentration of tubulin required for microtubule assembly, it does appear to stimulate microtubule nucleation (see “Discussion”).

Effect of Erythrocyte Tau on Microtubule Elongation

In Fig. 5 we show the specific effect of tau on microtubule elongation (as distinguished from nucleation) in a plot showing the relationship of the initial rate of change in turbidity (elongation) to tubulin subunit concentration. In this plot the slope and y intercept indicate the net assembly rate and net disassembly rate for microtubule growth while the x intercept indicates the critical tubulin concentration for assembly (see Ref. 11 for details describing the use of this plot). For subunits we used PC-tubulin supplemented to 5% with tau fraction similar to that shown in Fig. 2, lane G. Since this tau fraction was partially purified, the tubulin/tau mixture is actually comparable to a mixture containing 50% nontubulin fraction as shown in the preceding figure. We observed that partially purified tau slightly increased both the assembly rate and disassembly rate (slope and y intercept, respectively) and had only a slight effect on the critical concentration (x intercept

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**Fig. 2. Immunoblotting of microtubule protein from erythrocytes with tau antibodies.** A through F: Coomassie stain and immunoblot using Kirschner's affinity-purified rabbit antibody to hog brain tau. A, cycled chicken erythrocyte microtubule protein; B, erythrocyte extract; C, cycled chicken brain microtubule protein; D, chicken brain extract; E, cycled hog brain microtubule protein; F, hog brain extract. The asterisk and dash symbols indicate the major (67 kDa) and minor (70, 61, 56, 54 kDa) tau components in the erythrocyte microtubule protein sample. G through L: Coomassie stain and immunoblot using our affinity purified rabbit antibody to chicken erythrocyte 67 kDa tau. G, partially purified chicken erythrocyte tau; H, cycled chicken erythrocyte microtubule protein; I, chicken erythrocyte PC-tubulin; J, cycled chicken brain microtubule protein; K, cycled hog brain microtubule protein; L, partially purified hog brain tau. The asterisk and dash symbols indicate erythrocyte tau components as described above.
Erythrocyte Tubulin Assembly

Identification of tau as the assembly-promoting factor in the erythrocyte nontubulin protein fraction. Erythrocyte nontubulin protein (s, sample) was prepared from microtubule protein by phosphocellulose chromatography using elution with 1.0 M KCl. The asterisk and dash symbols indicate the major 67 kDa and minor tau components present in the sample as determined by immunoblotting (see Fig. 2). The preparation was fractionated by gel filtration chromatography on agarose A-1.5m (Bio-Rad) pre-equilibrated with assembly buffer. Aliquots of PC-tubulin (1.28 mg/ml final concentration) were added to each fraction and assembly was monitored by electron microscopy and by turbidity at 350 nm after 10 min at 37 °C. The composition of fractions as determined by SDS-polyacrylamide gel electrophoresis together with molecular weight identification of tau isoforms is shown in the upper panel.

nearly unchanged), which is in agreement with our observation that tau does not greatly alter the amount of polymer formed at steady state.

Tau Is Localized in the Marginal Band of Chicken Erythrocytes

The distribution of tau in chicken erythrocytes as determined by immunofluorescence microscopy is shown in Fig. 6. Tau antibody labels the marginal band of microtubules intensely and only weakly labels other regions of the cell. The qualitative impression is that tau may be exclusively localized in the marginal band in the extracted erythrocyte ghosts. We know from immunoblotting experiments using preparations of cytoskeletal and cytosolic proteins (defined by a Triton X-100 extraction procedure) that the majority of the tau is fixed in the cytoskeleton and is not soluble. Thus, tau, which copurifies with microtubules as do high M, MAPs and which also exhibits assembly-promoting activity in vitro, is associ-

D. B. Murphy and W. A. Grasser, manuscript in preparation.

Comparison of the Elongation Kinetics of Brain and Erythrocyte Tubulin

Having demonstrated the stimulatory effect of erythrocyte tau on both nucleation and elongation, we next compared the

FIG. 3. Identification of tau as the assembly-promoting factor in the erythrocyte nontubulin protein fraction. Erythrocyte nontubulin protein (s, sample) was prepared from microtubule protein by phosphocellulose chromatography using elution with 1.0 M KCl. The asterisk and dash symbols indicate the major 67 kDa and minor tau components present in the sample as determined by immunoblotting (see Fig. 2). The preparation was fractionated by gel filtration chromatography on agarose A-1.5m (Bio-Rad) pre-equilibrated with assembly buffer. Aliquots of PC-tubulin (1.28 mg/ml final concentration) were added to each fraction and assembly was monitored by electron microscopy and by turbidity at 350 nm after 10 min at 37 °C. The composition of fractions as determined by SDS-polyacrylamide gel electrophoresis together with molecular weight identification of tau isoforms is shown in the upper panel.

FIG. 4. Stimulation of tubulin self-assembly by erythrocyte tau. (PC-tubulin from erythrocytes was mixed at 5 °C with increasing amounts of erythrocyte nontubulin fraction (indicated as weight per cent). A, assembly at 37 °C was monitored by turbidity at 350 nm. The final tubulin concentration was 1.5 mg/ml. B, the extent and rate of assembly from the curves shown in A. The rate was calculated from the time required to reach one-half maximal turbidity.

FIG. 5. Effect of tau on tubulin elongation. Samples of PC-tubulin were supplemented to 5% with partially purified tau or with assembly buffer at 5 °C. The samples were then diluted with assembly buffer to the final tubulin concentrations indicated on the x axis. Subunits were preincubated at 30 °C for 50 s prior to the addition of exogenous microtubule seeds. (For details, see "Materials and Methods.") Microtubule seeds: brain microtubule protein.

ated with the microtubules in the marginal bands of permeabilized, extracted cells.
Fig. 6. The distribution of tau in chicken erythrocytes. Chicken blood was collected in citrate/saline (0.03% Na/citrate, 0.9% NaCl) and drops of the cell suspension were allowed to adsorb for 10 min on glass coverslips covered with 0.1 mg/ml polylysine. The coverslips were processed for immunofluorescence microscopy using our affinity-purified tau antibody as described by Osborn and Weber (33). In this procedure microtubules are stabilized with polyethylene glycol, and cells are extracted in Triton X-100 and "fixed" with cold methanol prior to incubation with antibody. In extracted cells, most of the tau is localized in the marginal band. Bar indicates 10 μM.

Published), the magnitude of this effect is small and does not affect the interpretation of the results presented here. We observed the following results.

(a) Microtubule Protein—Brain protein assembled with relatively greater assembly and disassembly rates than did erythrocyte protein; the critical concentrations were also similar (0.5 mg/ml for brain, 0.2 mg/ml for erythrocyte). These results agree with the general observation that brain protein assembles faster than erythrocyte protein (Fig. 1). However, unlike Fig. 1 which shows the combined effects of nucleation and growth during self-assembly, it can now be determined from this plot that brain microtubule protein gives a more rapid rate of subunit addition onto seeds and that both the on and off rate constants for brain protein are greater in magnitude by a factor of about 2.

(b) PC Tubulin—Erythrocyte PC-tubulin assembled with a faster rate than brain protein, just the opposite of what was observed for the microtubule protein from these tissues. The disassembly rates and critical concentrations of the preparations were similar. Part of the reason for the difference in relative elongation efficiencies is due to the absence of the stimulatory effect of high M, MAPs in the brain tubulin preparation (see c). Differential effects of phosphocellulose treatment on tubulin assembly competency are unlikely to be a major factor, because both the PC tubulin preparations were passed through a cycle of assembly and disassembly to select initial elongation rates for erythrocyte and brain tubulin. By comparing pairs of protein preparations we were able to evaluate the significance of MAPs and tubulin variants on elongation rates and determine as well the relative contribution of nucleation and elongation to the self-assembly process. Initial rate plots of microtubule elongation, prepared using microtubule protein and PC-tubulin as subunits, are shown in the four panels of Fig. 7. For each panel a pair of subunit preparations is compared using a single preparation of microtubule seeds (see legend). Before analyzing the figure, it is important to note that because the seed number concentration varies between different seed preparations, one cannot compare the values of slopes and intercepts between different panels; rather, only a relative comparison between preparations within a panel can be made. It should also be noted that certain mixtures of seeds and subunits resulted in the formation of heteropolymers of brain and erythrocyte tubulin. While some subtle differences in the rates of heteropolymer versus homopolymer formation were observed (±5%, data not published), the magnitude of this effect is small and does not affect the interpretation of the results presented here. We observed the following results.
for active subunits immediately prior to use. Further clarification of this observation is made in "d" and in the last section under "Results." This is the first observation that different tubulin isoforms elongate on microtubule seeds with different efficiencies in vitro.

(c) Brain Protein—The purification of brain tubulin on phosphocellulose selectively increases the magnitude of the dissociation rate and therefore increases the critical concentration. This effect was observed previously for microtubule protein from hog brain, from which it was concluded that MAPs stabilize microtubules by reducing the magnitude of the dissociation rate constant (11). The variability in the critical concentration of PC-tubulin from brain (0.2 mg/ml in "b," but 0.9 mg/ml in "c") is thought to be due to variability in the time that the brain subunits were kept on ice prior to assembly, a feature of the experimental design that was difficult to control. The erythrocyte PC-tubulin samples were consistently more stable and less variable than those from brain.

(d) Erythrocyte Protein—The purification of erythrocyte tubulin on phosphocellulose always increased the assembly efficiency of the protein, that is, PC-tubulin has a lower critical concentration than tubulin protein. This is just the opposite of what was observed for brain protein (c). The relationship of this pair of curves is also different from the pair of curves shown above in Fig. 5 (erythrocyte PC-tubulin + tau) where a stimulatory effect by tau was observed. These observations suggested that additional factors other than the presence or absence of tau may be important in determining the rate of assembly. It occurred to us that this difference could be due to the presence of tubulin oligomers, since erythrocyte microtubule protein is always very turbid due to the presence of rings, whereas the preparations of PC-tubulin are clear and do not contain rings.

Effect of Tubulin Oligomers on the Rate of Microtubule Elongation

Preparations of freshly prepared erythrocyte PC-tubulin were clear and did not contain rings or large oligomers as determined by electron microscopy but could be made to contain oligomers by passage through an additional cycle of in vitro assembly and disassembly (Fig. 8). The turbidity due to oligomers at 350 nm was usually of the order of 0.05 A for a 2 mg/ml sample of PC-tubulin after depolymerization at 0 °C. Generally, ring oligomers were not observed in preparations of disassembled PC-microtubules in assembly buffer containing 1 mM Mg²⁺, but the amount of turbidity and the presence of rings in depolymerized samples seemed to vary, depending on the batch of microtubule protein. (Ring oligomers were only consistently observed following the disassembly of microtubules in assembly buffer containing 2 mM Mg²⁺. At 10 mM Mg²⁺ large numbers of rings and flocculent aggregates of rings were observed. The rate of self-assembly of the ring-containing material was significantly retarded compared to the rate of assembly in 1 mM Mg²⁺, but the kinetics of self-assembly are complex and will require further investigation. In this paper we therefore confine our observations to the rate of elongation in assembly buffer containing 1 mM Mg²⁺.)

The rate of subunit addition onto seeds (elongation) was examined for preparations with and without the presence of tubulin oligomers (Fig. 9). We observed that preparations without oligomers assembled at a faster rate (greater slope) and at a slightly lower critical concentration than preparations containing oligomers. The estimated rate of depolymerization (y intercept) was the same for both samples. The reduction in elongation rate was shown not to be due to the

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**Fig. 8. Tubulin oligomers induced by the disassembly of microtubules containing PC-tubulin.** PC-tubulin prepared by phosphocellulose chromatography was negatively stained after a cycle of assembly and disassembly in assembly buffer containing 1 mM Mg²⁺. Short filamentous oligomers are observed. Magnification = 85,700. Bar = 100 nm.

**Fig. 9. Effect of tubulin oligomers on the initial rate of microtubule elongation.** PC-tubulin subunits without or with oligomers (prepared by a single cycle of assembly and disassembly) were compared for their efficiency in supporting microtubule elongation at 30 °C in assembly buffer containing 1 mM Mg²⁺. Microtubule seeds: brain microtubule protein. The net depolymerization rate (y intercept) was not changed, but the net polymerization rate (slope) was reduced.
addition of 0.5 mM GTP did not increase the rate, depletion of GTP or the accumulation of GDP, since the phenomenon was demonstrated to be reversible and was not due to protein denaturation or inactivation of the tubulin subunits. This conclusion is further supported by the observation that full assembly activity was retained after 4 cycles of assembly and disassembly. We conclude that it may be the tubulin oligomers themselves that retard the rate of subunit addition to microtubules during elongation (see “Discussion”).

We also compared assembly rates of ring-containing and ring-depleted preparations of microtubule protein made by high speed differential centrifugation (200,000 × g, 5°C, 120 min), but these experiments were generally inconclusive, since the supernatants rapidly regained turbidity at 5°C, even when care was used to remove only the top one-half of the supernatant. Although these supernatants did not contain rings, they did contain many filamentous oligomers similar to those shown in Fig. 5. The addition of aliquots of the ring-containing samples to ring-depleted material also did not alter the rate of microtubule self-assembly (data not shown). This result contrasts with the stimulatory effect of ring-containing preparations from brain microtubule protein (see “Discussion”). These observations support our previous observation (2) that erythrocyte tubulin has a great propensity to form oligomers at 5°C and also suggests that rings themselves do not act as nuclei for initiating polymerization.

**DISCUSSION**

Microtubules in the marginal bands of chicken erythrocytes are distinguished by their extreme length and stability (23). The studies presented here suggest that these properties are due to the presence of a microtubule-associated tau factor and to the unique characteristics of erythrocyte tubulin itself.

**Erythrocyte Tau Is Contained in the Marginal Band and Stimulates the Initiation of Microtubule Self-assembly in Vitro**—We identified tau in preparations of erythrocyte microtubule protein by its mobility on SDS gels, its heat stability, the distribution coefficient of native tau by gel filtration chromatography, and its cross-reactivity to two preparations of tau antibodies.

In previous studies on brain microtubules it was established that both tau and high M; MAPs co-purify with microtubules (8, 34), stimulate microtubule initiation when added to tubulin (5, 9–11, 34), and enhance the rate of addition of purified tubulin subunits onto microtubule seeds (10, 11, 31). In this study we demonstrate that tau stimulates nucleation (strongly) and elongation (weakly) of purified erythrocyte tubulin subunits, and that tau is localized specifically in the marginal band in Triton-extracted erythrocyte ghosts. However, the in vivo function of tau in microtubule assembly and marginal band formation and function is not clear.

The most obvious effect of tau on microtubule assembly in vitro is to stimulate nucleation, as evidenced by the shortening of the lag time prior to polymerization (self-assembly) in vitro (35). Similar properties are also exhibited by the high M; MAPs associated with brain microtubules (9, 11). However, the effect of tau is distinguished from the effects of high M; MAPs as follows: 1) erythrocyte tau stimulates tubulin elongation in a fundamentally different way than high M; MAPs. Tau increases the magnitude of both the net assembly rate and net disassembly rate but does not significantly shift the critical tubulin concentration for microtubule polymerization.

Thus erythrocyte tau increases the initial rate of elongation by increasing the net assembly rate and facilitating the addition of tubulin subunits. In contrast, chicken high M; MAPs preferentially decrease the disassembly rate but only slightly increase the assembly rate, with the overall effect being to decrease the critical concentration as was originally reported for hog brain high M; MAPs (11). The factor of increase in the elongation rate induced by 5% tau in a mixture of seeds and PC-tubulin subunits was approximately 30%. 2) Tau has a more significant effect on microtubule nucleation during self-assembly, with a 2-fold increase in tau causing a corresponding 2-fold increase in initiation rate as measured by the half-time required to reach the maximal extent of assembly over a broad range of tau concentrations. The effect of tau on nucleation is made more readily apparent if the assembly rates for tubulin preparations with and without added tau are compared. The corresponding factor of increase in the self-assembly rate due to enhanced nucleation was approximately 500% in preparations containing 5% tau. Roughly comparable relationships have been observed for nucleation in the presence of high M; MAPs (11).

Although the addition of tau to purified tubulin did not shift the critical concentration to a lower value, preliminary experiments indicate that tau may nevertheless stabilize microtubules in the presence of assembly buffer containing 25% glycerol (data not shown). When microtubules composed of PC-tubulin were diluted into glycerol-containing assembly buffer with a nontubulin protein fraction or preparations of purified tau, significant stabilization was observed compared with dilution into buffer without added factors. However, when the same experiment was performed with buffer containing only 5% glycerol, the effectiveness of tau in stabilizing microtubules was minimal. These observations suggest that tau may stabilize microtubules in vitro, depending on the solution conditions employed, and it is possible that tau may also stabilize microtubules in the marginal bands of erythrocytes in vivo. Although the effects of solvents have not yet been examined in detail, it remains a consistent observation that, in aqueous buffer lacking high concentrations of glycerol, the principal effect of tau on microtubule assembly is on nucleation rather than on elongation. As described below, however, we feel that part of the explanation for the stability of marginal band microtubules may be due to the properties of erythrocyte tubulin itself.

**Tubulin Isoforms Polymerize in Vitro with Different Levels of Efficiency**—As shown in Fig. 7B, erythrocyte tubulin is twice as efficient as brain tubulin in elongation off brain microtubule seeds. The use of cycled PC-tubulin allows one to compare the elongation rates of assembly-competent tubulin subunits in the absence of MAPs under identical solution conditions. Compared to brain tubulin, erythrocyte tubulin was observed to have a relatively greater assembly rate, but a similar disassembly rate, and hence a lower critical concentration. If one could perform an experiment to compare the relative stabilities of these polymers, such as by dilution into assembly buffer, one would predict that the erythrocyte polymers would display greater stability. This is the first demonstration that biochemically distinct tubulin isoforms assemble in vitro with different levels of efficiency. It is a significant observation, since it gives credence to the hypothesis that different tubulin isoforms may exhibit different properties in the cell, thus providing a mechanism for the regulation of microtubule assembly and function.

**Microtubule Assembly Occurs at a Reduced Rate in the Presence of Tubulin Oligomers**—We demonstrated that the rate of tubulin addition onto microtubule seeds during elon-
gation is reduced when the tubulin pool used for elongation is in the form of oligomers. In this section we discuss this idea together with an idea first presented by Weisenberg (36) that a storage form of tubulin may be used to control tubulin utilization in the cell.

In a previous report we described the tendency of erythrocyte microtubule protein to form ring oligomers at 5°C; 40% of the protein in preparations of microtubule protein at 1-2 mg/ml is in the form of large ring-containing aggregates and can be pelleted by low-speed centrifugation (40,000 × g, 20 min, 5°C). In this report we now show that the presence of oligomers reduces the rate of microtubule elongation in vitro and that the addition of ring-containing protein to ring-depleted preparations did not stimulate self-assembly. We propose that this fact is partly the reason why erythrocyte microtubule protein self-assembles so slowly in vitro. The reduced rate of elongation in the presence of rings confirms previous findings that microtubule elongation at elevated temperatures near 30°C occurs primarily through dimer addition and not by oligomer addition (24) and that tubulin ring oligomers do not directly initiate tubulin assembly and are not directly incorporated into microtubules during elongation (24, 37). The issue of rings and assembly is somewhat complicated by the fact that recent reports show that small oligomers of tubulin, possibly derived from rings, may act as polymerizing units during self-assembly (37-39), as was proposed earlier in a theoretical model by Weisenberg (40).

If elongation through subunit addition is correct, the dissociation of tubulin oligomers on phosphocellulose would be expected to increase the concentration of tubulin dimers and therefore increase the initial rate of tubulin elongation on microtubule seeds. The general equation posited for the rate of microtubule elongation (11) is:

\[
\frac{dA}{dt} = -k[S][M] + k'[M]
\]

where [S] and [M] indicate the concentrations of subunits and microtubules, respectively. One would predict that the effect of phosphocellulose treatment, (increasing the tubulin dimer concentration) would be to increase the polymerization rate, \(k'\), which is dependent on [S], but not the depolymerization rate, \(k\), which is independent of [S], and this is what was observed in Fig. 9.

The big surprise is that any effect of tubulin oligomers was observed at all. Oligomers of brain microtubule protein dissociate rapidly at temperatures that permit microtubule assembly, and it has generally been difficult to observe their effect on polymerization (41). It is likely that the difference is due to the erythrocyte tubulin isoform whose unique biochemical properties have already been described.

It is appropriate to make an additional comment on the earlier observations by Olmsted et al. (42) and Weingarten et al. (5) who reported that preparations of microtubule protein depleted of rings exhibited reduced rates of self-assembly. Since the addition of ring-containing material to these preparations again restores the rapid assembly rate it appears that ring preparations of brain microtubule protein contain an activity that stimulates microtubule nucleation and is responsible for the rapid rates of self-assembly. Our results using erythrocyte tubulin argue that the tubulin oligomers themselves do not contain nucleating activity and that they retard the rate of elongation. We suggest that it may have been some other factor such as MAPs or certain MAP-tubulin complexes that actually stimulated self-assembly in those experiments. Recent evidence suggests that this may have been the case (37-39).

Our observations on the negative effect of tubulin oligomers on assembly rates may be directly related to the mechanism for microtubule assembly and marginal band formation in vitro. One remarkable feature about marginal band microtubules is their extremely long length. Euteneuer et al. (43) have suggested the marginal band contains only a few microtubules and Nachmias (44) has described a single "hoop of microtubule" (one microtubule coated many times around the cell circumference) in mammalian platelets. While the exact number of microtubules is not known, it is generally agreed that the microtubules in the marginal band are long. It is challenging to consider how this structure is formed in the cell, since many microtubules would be expected to self-assemble at the onset of band formation. Kravit et al. (45) recently discussed the idea that the regulation of a tubulin oligomer pool in cells may be important for controlling microtubule growth of microtubule organizing centers as well as tubulin gene expression. One could likewise consider the idea, that a tubulin oligomer pool in the erythrocyte may be important for maintaining low dimer concentrations and hence low nucleation rates in order to generate long microtubule lengths. The idea of a tubulin oligomer storage form in cells was originally proposed by Weisenberg (40, 46). Intracellular control of microtubule growth by a tubulin oligomer pool could occur if the equilibria governing subunit association with oligomers and microtubule polymers were similar in magnitude, and recent evidence shows that this might be the case (41). In a previous report (2) we made an independent observation that points to this possibility. Erythrocyte ghosts extracted and washed in solutions containing Triton contain twice as much tubulin as can be accounted for in their marginal bands, and intact erythrocytes contain a "soluble" tubulin pool whose concentration is five times greater than that observed for the critical concentration for tubulin assembly in vitro. A similar observation was made regarding the soluble tubulin pool in neuroblastoma cells (47). The form of this non-band, non-extractable tubulin in erythrocyte ghosts and the large pool of soluble tubulin in intact cells is not known, but it remains an interesting possibility, and at this time still a speculation, that it consists of tubulin oligomers and that the dynamic activity of microtubules may be regulated by the activity of a tubulin oligomer pool.

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