Characterization of a 20S Component in Tubulin from Mammalian Brain†

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ABSTRACT: Tubulin from porcine brain, purified by at least two cycles of assembly and disassembly, was characterized at different pH values by sedimentation velocity analysis and turbidimetric measurements. At pH 6.4 the depolymerized material was composed of two major species sedimenting with $s^0_{20,w}$ values of 6 and 36 and a minor one of 20 S. By raising the pH, the amount of the 20S component increased and that of the 36S decreased, whereas that of the 6S component was unaltered. At pH 7.6 the mixture contained 20S and 6S components but hardly any 36S. The 20S species can be separated from the 6S ones by gel filtration on agarose A-15m at pH 7.6. On electron microscopic examination this preparation contains far fewer double rings compared to the material at pH 6.4, but single rings could often be seen. Sodium dodecyl sulfate gel electrophoresis of the 20S and 36S components showed that they consist almost entirely of tubulin and some higher and lower molecular weight fractions. Turbidity measurements showed that the minimal protein concentration necessary for polymerization increases with increasing pH. The turbidity plateau reached at a given pH can be raised by decreasing the pH. From these results it is suggested that the 20S component is an intermediate of the 36S species. The results further indicate the existence of a pH-dependent equilibrium between the 20S species and the 36S oligomers.

Microtubules are involved in a wide variety of cellular processes and serve essential functions in the maintenance of cell form, formation of the spindle apparatus in mitosis, transport of material in the cell, neurotransmission in nerve axons, and ciliary and flagellar motility (Porter, 1966; Tilney, 1971; Kreutzberg, 1969; Olmsted and Borisy, 1973; Satir, 1968).

Until recently, detailed information on the conditions necessary for microtubule assembly and disassembly was limited. The first conditions for the in vitro assembly of microtubules in brain cytosol came from Weisenberg (1972) and Borisy and Olmsted (1972). Shelanski et al. (1973) developed a procedure for isolating large amounts of tubulin by using these conditions. Both Borisy and Olmsted (1972) and Shelanski et al. (1973) found that assembly required the presence of nucleation sites. Kirschner et al. (1974) and Weingarten et al. (1974) demonstrated the presence of 6S and 36S particles in depolymerized microtubules. When the separated species were analyzed, it was found that only the 36S component would form microtubules but not the 6S alone. Examined by electron microscopy the 36S component consists mainly of double rings and spirals. More recently Weingarten et al. (1975) reported that the ability of 36S rings to assemble in microtubules is due to a heat-stable protein, called τ, which is present in the 36S species but is absent in the 6S dimer. Bryan et al. (1975) found that spontaneous assembly of microtubules in nonneural cell extracts is blocked because the endogenous τ factors are complexed with an inhibitor (RNA or other polyanions).

In a preliminary report we presented evidence that purified microtubule protein preparations contain the 6S and 36S components and a third 20S species (Doenges et al., 1975). In the present paper the 20S component is characterized in more detail.

Materials and Methods

Tubulin Preparation. Porcine brain tubulin was prepared with slight modifications of the procedure of Shelanski et al. (1973). Routinely, 500–600 g (wet weight) of fresh brain with the blood vessels and meninges dissected was homogenized in 500–600 ml of ice cold buffer (0.1 M Mes,[1] 0.5 mM $MgCl_2$, pH 6.4) in an Ultraturrex homogenizer. The homogenate was initially centrifuged at 20 000g for 45 min at 4 °C and the supernatant recentrifuged at 100 000g for 60 min at 4 °C. This supernatant was diluted 1:1 with buffer containing 8 M glycerol, 2 mM GTP, warmed to 37 °C for 45 min and then centrifuged at 100 000g for 60 min at 30 °C. The pellets were resuspended in one-tenth volume of prewarmed (20 °C) buffer (without GTP and glycerol) by gentle homogenization in a Dounce homogenizer, chilled on ice for 30 min, and centrifuged at 100 000g for 60 min at 4 °C. The tubulin containing solution was made up to 4 M glycerol, stored at −20 °C, and used within 2 weeks.

In experiments involving tubulin solutions of different pH values a suitable sample of the protein solution was removed, GTP was added to 1 mM, the solution was warmed up to 37 °C for 45 min, and the microtubules were collected by centrifugation at 100 000g for 60 min at 30 °C. The pellets were resuspended in buffer (20 °C) of the desired pH; the suspension was cooled at 0 °C for 30 min and clarified by centrifuging at 100 000g for 60 min at 4 °C. In experiments to be done in the presence of Ca^{2+}, Mes buffer supplemented with 2 mM $CaCl_2$ was used to resuspend the pellets.

Protein concentrations were determined by the method of Lowry et al. (1951). Polyacrylamide-sodium dodecyl sulfate gel electrophoresis was carried out according to the method of Shapiro et al. (1967). Polymerization of microtubules was measured by using the turbidity assay developed by Gaskin et al. (1974). The measurements were made at 350 nm; microtubule assembly was induced by elevating the temperature of tubulin extracts from 0 to 37 °C.

Sedimentation velocity experiments were done using a preparative Beckman ultracentrifuge Model L 75-B, equipped with a schlieren optics accessory. $s^0_{20,w}$ values were determined for samples sedimented in 12-mm single sector cells with plain

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[1] Abbreviation used is: Mes, *N*-morpholinoethanesulfonic acid.

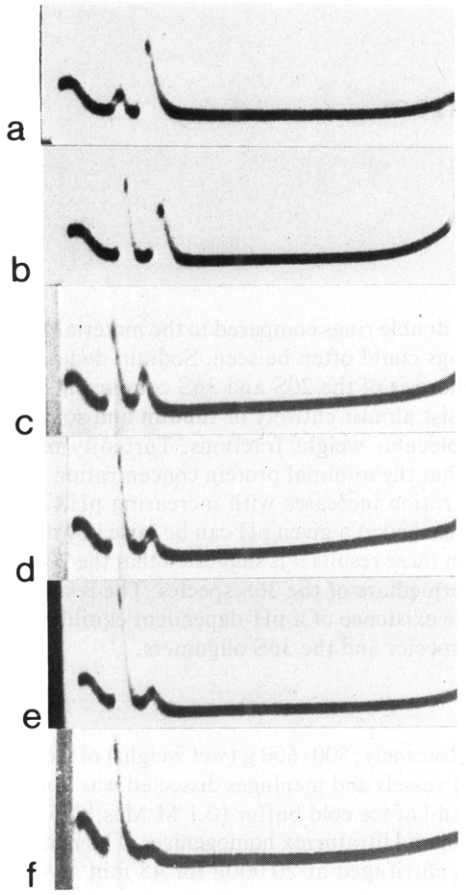

FIGURE 1: Sedimentation patterns of purified tubulin preparations at pH values of 6.4, 6.7, 6.9, 7.1, 7.3, and 7.6 from top to bottom (a–f). Purified microtubule protein was prepared in Mes–Ca^{2+} buffer of the desired pH as described in Materials and Methods. Samples (5–6 mg of protein/ml) were sedimented at 20 °C at 50 000 rpm. Photographs were taken 20 min after reaching maximal speed; the schlieren phase plate was set at an angle of 70°.

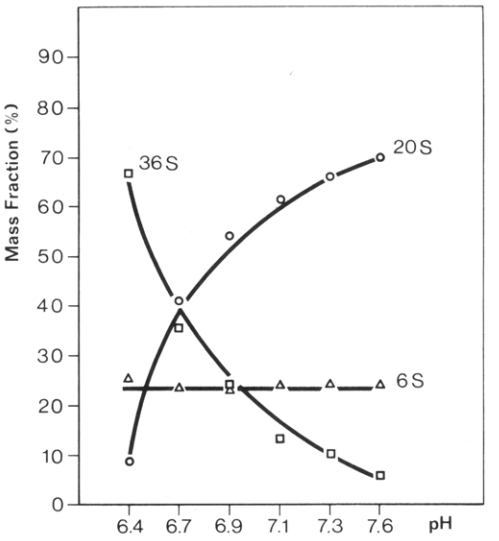

FIGURE 2: Analytical ultracentrifugation analysis of the 36S, 20S, and 6S species as a function of pH. The mass fraction of each sedimenting component was determined by planimetry of the schlieren optic peaks.

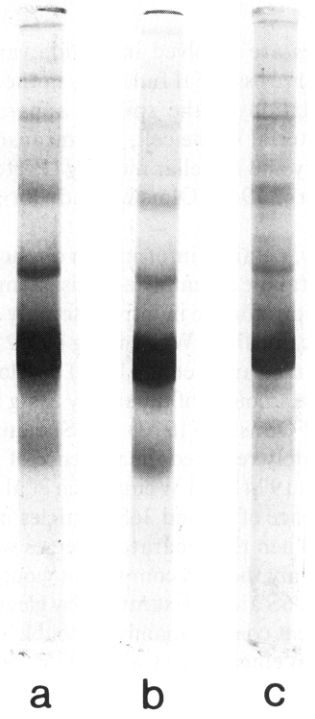

FIGURE 3: Sodium dodecyl sulfate–polyacrylamide gels of the fractionated 6S, 20S, and 36S species. The 36S and 20S components were separated from the 6S component by chromatography on agarose A 15m at pH 6.4 and 7.6: (a) leading fraction (20 S) from agarose column at pH 7.6; (b) leading fraction (36 S) from agarose column at pH 6.4; (c) trailing fraction (6 S) from agarose column at pH 6.4. The gels were intentionally overloaded to bring out the minor components.

windows by extrapolation of the sedimentation coefficients to infinite dilution. The mass fraction of each sedimenting component was determined by planimetry of the schlieren photographs, correcting for radial dilution. All experiments were done with an analytical An-D rotor at a rotor speed of 50 000 rpm.

Fractionation of the 36S components from the 6S ones was performed with microtubule protein solutions of concentrations between 20 and 25 mg/ml in Mes–Mg buffer containing 2 mM $CaCl_2$ at pH 6.4. The solution (3 ml) was applied to a 50 cm × 1.5 cm column containing Bio-Gel A-15m, 100–200 mesh (Bio-Rad). The elution buffer was Mes–Mg, 2 mM $CaCl_2$ at pH 6.4. The fractionation of the 20S species from the 6S ones was done in the same way by using similar protein concentrations in buffer of pH 7.6.

Samples for electron microscopy were prepared by negative staining with uranyl acetate. One drop from each sample was applied to a carbon-coated grid for 30 s and stained by rinsing with 2 drops of 2% uranyl acetate and drying with filter paper.

Results

Components in Tubulin Preparations of Different pH Values. Purified microtubule protein preparations in Mes–Ca^{2+} buffer of pH values between 6.4 and 7.6 were analyzed by analytical ultracentrifugation. As shown in Figure 1a, samples of purified material at pH 6.4 were composed of two major species of about 6 and 36 S and a minor one of about 20 S. With increasing pH the amount of the 36S component decreases and that of the 20S species increases (Figures 1b–1f). At pH 7.6 the 6S and 20S species are predominant and only a minor one of 36S is seen (Figure 1f). Using samples at the same protein concentrations, the relative proportions of the 6S, 20S, and 36S components as a function of pH were determined by planimetry of the schlieren optic photographs. Figure 2

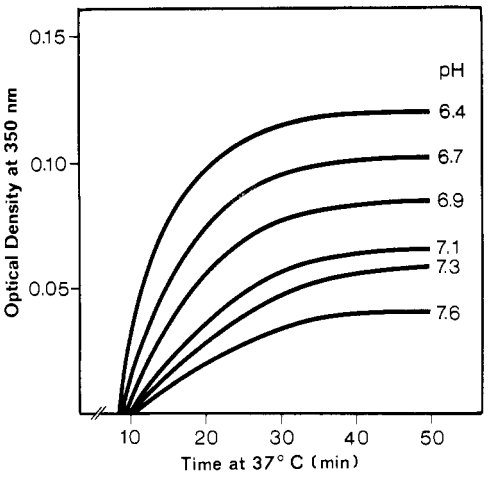

FIGURE 4: Effect of pH on the time course of microtubule assembly monitored using turbidity measurements at 350 nm and 37 °C. Polymerization of microtubules was induced by addition of 1 mM GTP to protein solutions (1 mg/ml) and elevating the temperature from 0 to 37 °C.

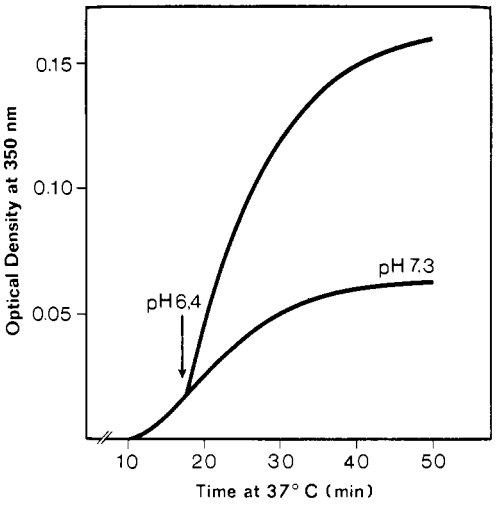

FIGURE 5: Reversibility of the pH-dependent tubulin polymerization. Two samples of tubulin (1 mg/ml) in assembly buffer at pH 7.3 were polymerized at 37 °C. Shortly after the assembly had started, the pH of one sample was adjusted to pH 6.4 (arrow); the pH of the other one was unaltered.

shows that at pH 6.4 the 36S peak represents 70% of the total protein, whereas that of the 20S is 10%. At pH 7.6, 70% of the 20S and only 10% of the 36S component is in this fraction. The amount of the 6S species seems to remain constant between pH 6.4 and 7.6. Equivalent results were obtained either if microtubule pellets were resuspended at a given pH or if a single preparation of tubulin was titrated to a given pH.

From these results it is suggested that the 36S and the 20S components are in a pH-dependent equilibrium.

Sodium Dodecyl Sulfate Gels of 6S, 20S, and 36S Proteins. The 20S and 36S components were separated from the 6S ones as described under Materials and Methods and analyzed by gel electrophoresis. The results are shown in Figure 3. Tubulin is the predominant component in cycled microtubule preparations; however, a number of other nontubulin proteins are always present. A similar finding has now been reported by Sandoval and Cuatrecasas (1976). It is not yet clear if all of these proteins are essential for assembly. Separated 6S, 20S, and 36S fractions show the same electrophoretic pattern. On

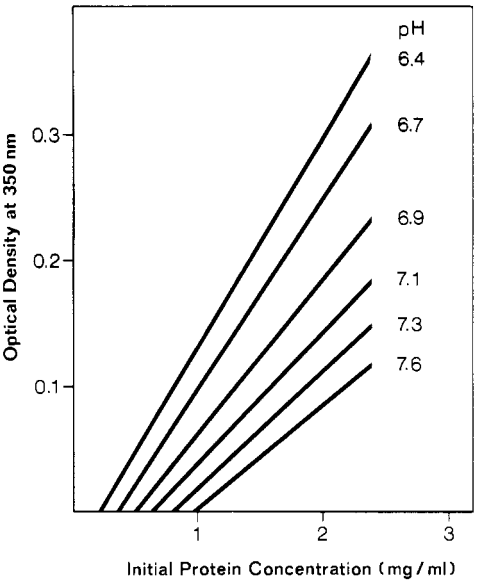

FIGURE 6: Dependence of the initial protein concentration on pH. The microtubule protein solutions were preparations in Mes-Mg buffer of the desired pH. The actual points have been left out for the sake of clarity.

the basis of these criteria it is apparent that the 20S fraction is like the 36S component an oligomer of tubulin.

Turbidimetric Characteristics for Tubule Assembly at Different pH Values. Gaskin et al. (1974) have shown that turbidity is a reliable measure of the mass of tubulin assembled into microtubules. The time course of appearance of turbidity is a function of the protein concentration and below a critical concentration, C_c, no assembly occurs. Above C_c the turbidity begins to increase and is linear with the protein concentration. C_c is increased by the addition of anti-tubulins, Na^+ and Ca^{2+} ions, and one possibility is that C_c can be varied by altering the relative concentration of the 36S component (Kirschner et al., 1974; Erickson, 1974; Olmsted et al., 1974; Bryan and Nagle, 1976).

To correlate the pH-dependent changes of the amounts of the 36S and 20S species with the effect of pH on tubule assembly and on C_c, we examined the time course of polymerization at 37 °C as a function of pH (Figure 4). By increasing the pH in the range between 6.4 and 7.6 the initial rates of polymerization and the plateau values of the optical density (OD) at 350 nm are decreased. Adjusting a solution of pH 7.3 to pH 6.4 shortly after the assembly had started results in an immediate increase of the polymerization rates, showing that the pH-dependent polymerization reaction is reversible (Figure 5).

In order to demonstrate the effect of pH on C_c, turbidity measurements at 350 nm were done in samples of various tubulin concentrations at different pH values. As shown in Figure 6, by increasing pH values C_c is also increased.

From these results it is concluded that by increasing pH values the decrease of the 36S component causes a decrease in microtubule assembly and an increase of C_c.

Electron Microscopic Observations on Depolymerization Products. The results of sedimentation velocity analysis at different pH values suggested that a structure other than double rings and spirals (36 S) and dimers (6 S) may exist in depolymerized microtubules. Therefore, electron microscopic examinations on depolymerized products at pH 6.4 and 7.6 were made to determine the morphological structures.

Material from the leading fractions from an agarose column isolated at pH 6.4 contained characteristic double ring struc-

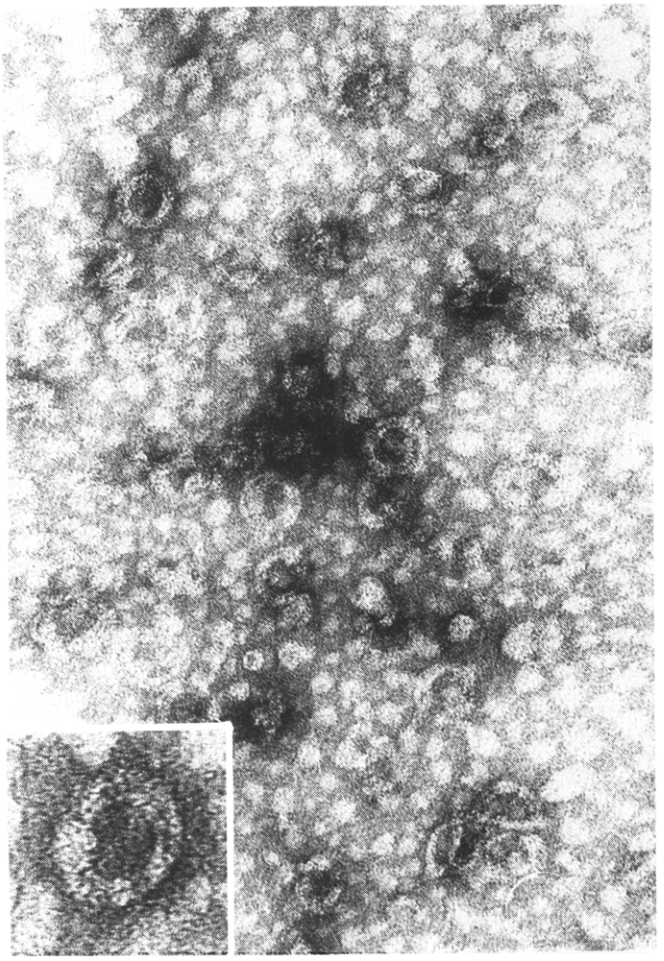

FIGURE 7: Electron micrograph of double rings in Mes-Ca^{2+} buffer at pH 6.4. The sample was negatively stained with 2% uranyl acetate; ×200 000; inset ×400 000.

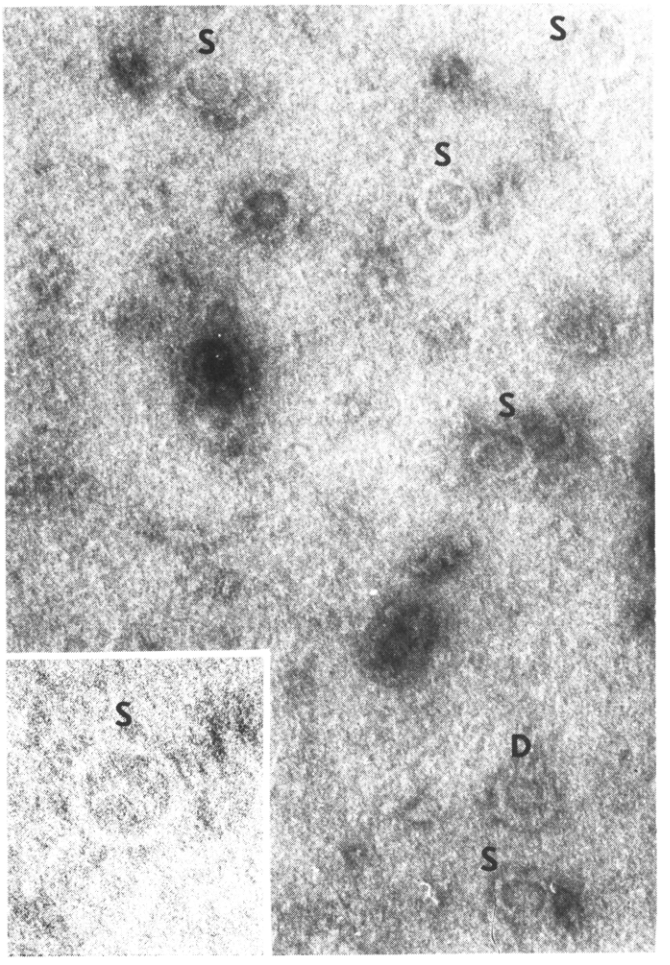

FIGURE 8: Electron micrograph of single rings (S) in Mes-Ca^{2+} buffer at pH 7.6. Some double rings (D) are also seen. Negative staining with 2% uranyl acetate; ×200 000; inset, ×400 000.

tures which were already described by Kirschner et al. (1974) and Erickson (1974, 1975) (Figure 7). The average outer diameters of the double rings in our preparations are 40 nm and 30 nm for the inner ring, determined by size measurements on about 80 double rings. Most of the rings can be seen as double rings with the same transition forms between double rings and spirals. In some areas annealing of the rings to form short chains could be found and in only a few cases could single rings be seen.

When the 20S species was separated on an agarose column at pH 7.6 the appearance of the structures differed from that of the fraction at pH 6.4. The majority of the structures exhibited single rings; the minority were double rings (Figure 8). Size measurements made on more than 60 single rings in several preparations yielded a mean diameter of 40 nm which corresponds to the outer diameter of the double rings.

Discussion

The results demonstrate that material from depolymerized brain microtubules contains three components, one with a sedimentation coefficient of 6 S and two others with coefficients of 36 and 20 S. The 20S species is mainly formed at pH 7.1–7.6, whereas the 36S component predominates at pH 6.4. There exists a pH-dependent equilibrium between the 20S and the 36S species.

Electron microscopic examination of material at pH 6.4 revealed double rings with an average outer diameter of 40 nm. Kirschner et al. (1974) and Erickson (1974, 1975) described double rings with outer diameters of 43 and 42 nm which are comparable to the rings we measured. Weingarten et al. (1974), however, presented a model for double rings with outer diameters of 48.6 nm and Borisy and Olmsted (1972) found disks (30S components) with diameters of 35 nm. Preparations at pH 7.6 showed under the electron microscope mainly single rings which obviously represent the 20S component found by sedimentation velocity analysis at this pH. These findings are supported by theoretical calculations of Weingarten et al. (1974) who predicted that single rings would sediment at 21 S. Kirschner et al. (1974) found in depolymerization products of microtubules at pH 6.4, in addition to double rings, a few single rings (less than 10%) and Olmsted et al. (1974), Borisy et al. (1975), and Rebhun et al. (1975) observed in purified microtubule protein preparations above pH 6.9 an additional component of 20 S in addition to 6S and 36S species.

Using column chromatography the 20S component was separated from the 6S species at pH 7.6 and the 36S component was fractionated at pH 6.4. The leading fraction at pH 6.4 contained mainly the 36S component and the one at pH 7.6 the 20S species, the trailing fractions being solely 6S material. On sodium dodecyl sulfate–acrylamide gel electrophoresis the leading and the trailing fractions show the same electrophoretic pattern of tubulin and some higher and lower molecular weight bands. The intentionally overloaded gels demonstrate the presence of nontubulin proteins after several cycling purifi-

cations. The findings are similar to reports of Sandoval and Cuatrecasas (1976) and also of Kuriyama (1975) and Tatsuya and Kurokawa (1975). Borisy et al. (1974) and Murphy and Borisy (1975) have found that the only proteins that copurify with tubulin are high molecular weight species. Weingarten et al. (1975) did not detect any major polypeptides, either in the leading or in the trailing fraction. The role of tubule-associated proteins remains to be elucidated.

The decrease of the initial rates of tubulin polymerization and of the plateau values of $OD_{350\ nm}$ by increasing the pH range between 6.4 and 7.6 shows that the 20S component is present under conditions in which polymerization is not maximal. These data and the observation that by increasing pH the critical protein concentration for tubulin polymerization is increased suggest that the 20S aggregates are not as effective as the 36S species and that they are not an assembly intermediate.

Kirschner et al. (1974) and Weingarten et al. (1974, 1975) proposed a model for tubule assembly based on tubulin in two states, free 6S tubulin subunits (X tubulin) and 36S double rings or spirals composed of 6S subunits (Y tubulin). Only the 36S rings will assemble into tubules; the free 6S subunits will not form rings or tubules. The inability of free 6S tubulin to form microtubules was explained by the absence of the τ factor, a protein essential for microtubule assembly.

We postulate that tubulin subunits from mammalian brain may occur in a third state which can be termed "Z". The Z-state molecules occurring in the 20S species seem to be unable to polymerize into microtubules. Preliminary results show that the 20S component also contains a protein factor which can be removed from tubulin by chromatography on phosphocellulose at pH 7.6. It is not yet clear if this protein factor is identical with τ and why the 20S components are not as effective in tubulin assembly as the 36S species.

The presence of the 20S component in depolymerization products of microtubules has been shown to be not as essential for tubule assembly in vitro as the 36S species. However, we believe that the 20S material could offer biological advantages in vivo. The cell could synthesize large amounts of tubulin and hold them reserved as 20S species. The equilibrium between the 36S and the 20S structures could be regulated by small pH differences within the cell. It is possible that the 20S component could serve as storing material which the cell could use if synthesis of microtubules is required.

Acknowledgments

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References

Borisy, G. G., and Olmsted, J. B. (1972), *Science 177,* 1196.

Borisy, G. G., Marcum, J. M., Olmsted, J. B., Murphy, D. B., and Johnson, K. A. (1975), *Ann. N.Y. Acad. Sci. 253,* 107.

Borisy, G. G., Olmsted, J. B., Marcum J. M., and Allen, C. (1974), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* 167.

Bryan, J., and Nagle, B. W. (1976), in the 26th Mosbach Colloquium, Molecular Basis of Motility, Heilmeyer, F. C., Rüegg, F. C., and Wieland, Th., Ed., West Berlin and Heidelberg, W. Germany, Springer-Verlag, p 161.

Bryan, J., Nagle, B. W., and Doenges, K. H. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3570.

Doenges, K. H., Biedert, S., and Paweletz, N. (1975), *J. Cell Biol. 67,* 97a.

Erickson, H. P. (1974), *J. Supramol. Struct. 2,* 393.

Erickson, H. P. (1975), *Ann. N.Y. Acad. Sci. 253,* 60.

Gaskin, F., Cantor, C. R., and Shelanski, M. L. (1974), *J. Mol. Biol. 89,* 737.

Kirschner, M. W., Williams, R. C., Weingarten, M., and Gerhart, J. C. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1159.

Kreutzberg, G. W. (1969), *Proc. Natl. Acad. Sci. U.S.A. 62,* 722.

Kuriyama, R. (1975), *J. Biochem. 77,* 23.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Murphy, D. B., and Borisy, G. G. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2696.

Olmsted, J. B., and Borisy, G. G. (1973), *Annu. Rev. Biochem. 42,* 507.

Olmsted, J. B., Marcum, J. M., Johnson, K. A., Allen, C., and Borisy, G. G. (1974), *J. Supramol. Struct. 2,* 429.

Porter, K. (1966), in Principles of Biomolecular Organization, Wolstenholme, G. E., and O'Connor, M., Ed., Boston, Mass., Little, Brown and Co., p 273.

Rebhun, L. A., Jemiolo, D., Ivy, N., Mellon, M., and Nath, J. (1975), *Ann. N.Y. Acad. Sci. 253,* 362.

Sandoval, I. V., and Cuatrecasas, P. (1976), *Biochem. Biophys. Res. Commun. 68,* 169.

Satir, P. (1968), *J. Cell Biol. 39,* 77.

Shapiro, A., Vinuela, E., and Maizel, J. (1967), *Biochem. Biophys. Res. Commun. 28,* 815.

Shelanski, M. L., Gaskin, F., and Cantor, C. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 765.

Tatsuya, H., and Kurokawa, M. (1975), *Biochim. Biophys. Acta 392,* 335.

Tilney, L. G. (1971), in Origin and Continuity of Cell Organelles, Reinert, J., and Ursprung, H., Ed., New York, N.Y., Springer-Verlag, p 222.

Weingarten, M. D., Lockwood, A. H., Hwo, S., and Kirschner, M. W. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1858.

Weingarten, M. D., Suter, M. M., Dittman, D. R., and Kirschner, M. M. (1974), *Biochemistry 13,* 5529.

Weisenberg, R. C. (1972), *Science 177,* 1104.