Properties of the Depolymerization Products of Microtubules from Mammalian Brain†

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ABSTRACT: Preparations of microtubules, isolated from porcine brain by a method of repeated polymerization and depolymerization, consist of 95% pure α and β tubulin, as judged by sodium dodecyl sulfate gel electrophoresis. Depolymerization of these tubules, either by addition of 1 mM $CaCl_2$ or by cooling to 4°, produces two protein components with $s_{20,w}^0$ of 6 and 36 S, as judged by analytical ultracentrifugation. The proportion of protein in the 36S form varies from 30 to 80% depending on the total protein concentration and the method of depolymerization. The depolymerized mixture can be resolved into two fractions by gel permeation chromatography. The leading fraction contains the 36S component, which forms 80% of the protein, and a 6S component which forms 20% of the protein. The trailing fraction is composed of only 6S protein. Sodium dodecyl sulfate gel electropherograms of the protein in either fraction indicate that they both consist almost entirely of α and β tubulin. The 36S component can be broken down to a 6S form under a number of conditions, such as addition of 0.75 M NaCl. Upon removal of the excess salt by dialysis the 36S component re-forms spontaneously. Re-formation of the 36S component is insensitive to 1 mM colchicine. An estimate of the molecular weight of the 36S component is obtained by a combination of sedimentation and electron microscopic data. Possible ways in which the 6S and 36S components could arise from microtubules are discussed, and a model is proposed in which microtubules contain two different types of 6S subunits, one of which will spontaneously aggregate into a 36S structure.

The study of microtubules by electron microscopy began over a decade ago. Cytoplasmic microtubules are recognized as long, hollow cyclinders about 25 nm in diameter (Stephens, 1971; Olmsted and Borisy, 1973) and appear to be involved in cell migration, formation of the spindle apparatus in mitosis, and the maintenance or modification of cell shape, as in neuron elongation. As major components of cilia and flagella, microtubules are also important in cell motility.

Conditions which allow the polymerization of microtubules in crude brain extracts were recently worked out by Weisenberg (1972) and Borisy and Olmsted (1972). Shelanski *et al.* (1973), using these conditions, developed a scheme for the purification of microtubules from guinea pig brain. Microtubules purified in this manner consisted of 95% pure tubulin, and could be repeatedly depolymerized by cooling and repolymerized by warming, as judged by electron microscopy.

Both Borisy and Olmsted (1972) and Shelanski *et al.* (1973) demonstrated that polymerization of microtubules was prevented if the depolymerized mixture was first centrifuged at high speed, even though much of the protein remained in the supernatant. Polymerization would occur, however, if a small amount of the high-speed pellet was added back to the supernatant. They proposed that the high-speed pellet contained a "nucleating center" which was required for polymerization. It became clear, at this point, that in order to understand the process of microtubule polymerization, it was necessary to describe more fully, first, what components were present in a depolymerized mixture of microtubules, and, second, how these components were utilized, if at all, in polymerization.

Recently, by ultracentrifugation of depolymerized microtubules, we have demonstrated the presence of two components which sediment as 6S and 36S species, and which together account for at least 85% of the protein in the mixture (Kirschner *et al.*, 1974). The 6S component does not polymerize in the absence of the 36S component. Examined by electron microscopy, the 36S component consists mainly of double-ring and spiral structures which appear to be formed by coiling of the microtubule protofilaments during depolymerization.

In this paper, we describe in detail some of the properties of the 6S and 36S components and how they interact, and consider the possible ways in which they could arise from microtubules.

Experimental Section

Microtubules were purified by the method of Shelanski *et al.* (1973) with slight modification, as follows. Brains from freshly slaughtered hogs were supplied by a local slaughterhouse. The superficial blood vessels and meninges were removed and the brains homogenized in 0.75 ml of purification buffer (0.1 M Mes,[1] 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM mercaptoethanol, 0.1 mM EDTA, and 1 mM GTP, pH 6.4) per gram of brain tissue. Homogenization was performed in the cold in a Waring Blendor. The homogenate was centrifuged at 45,000*g* for 2.5 hr at 4° in a Beckman Type 21 rotor. The supernatant was removed, diluted 1:1 with purification buffer containing 8 M glycerol, and warmed to 37° for 20 min to allow microtubule polymeriza-

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[1] Abbreviations used are: MES, *N*-morpholinoethanesulfonic acid; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid, disodium salt.

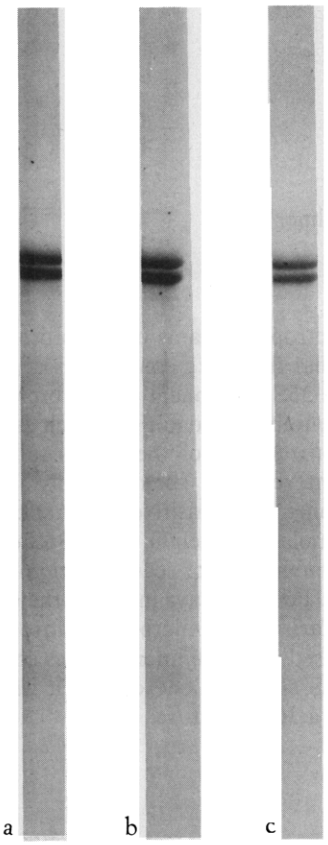

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of microtubule fractions: (a) microtubule protein purified by three cycles of polymerization–depolymerization; (b) leading fraction from agarose column of Ca^{2+}-dissociated microtubules; (c) trailing fraction of agarose column.

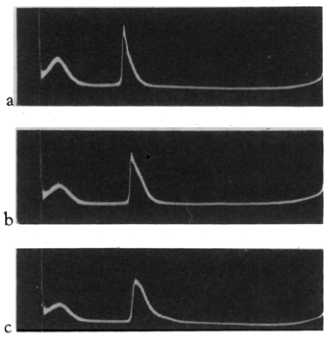

FIGURE 2: Ultracentrifuge patterns of depolymerized microtubules. Microtubules were dialyzed for 18 hr *vs.* two changes of 0.1 M Mes buffer containing 0.5 mM $MgCl_2$ and 1 mM EGTA (pH 6.4) at 4°. The protein was diluted to 7.5 mg/ml and examined in the ultracentrifuge under the following conditions. (a) At 0° with the addition of 1 mM GTP. The photograph was taken 28 min after reaching an operating speed of 47,660 rpm. (b) At 25° in the absence of GTP. The photograph was taken 16 min after reaching an operating speed of 47,660 rpm. (c) At 25° with the addition of 2 mM $CaCl_2$ and 1 mM GTP. The photograph was taken 16 min after reaching an operating speed of 47,660 rpm. In each experiment, the schlieren phase plate was set at an angle of 75°. The three photographs were taken at approximately identical effective sedimentation times.

tion. This was centrifuged at 45,000*g* for 2.5 hr at 25°, and the supernatants were discarded. The pellets were resuspended in 0.2 ml of purification buffer per gram of brain, chilled on ice for 30 min, and centrifuged in the cold at 75,000*g* for 75 min in a Beckman Type 30 rotor. The pellets from this centrifugation were discarded. The supernatants were diluted 1:1 with purification buffer containing 8 M glycerol and warmed to 37° for 20 min. This microtubule suspension was centrifuged at 25° for 75 min at 75,000*g*. The pellets, containing the purified microtubule protein, were suspended in cold purification buffer, and were then made up to 8 M glycerol by addition of pure glycerol, so that the final concentration of protein was 3.5–6 mg/ml.

The protein was stored at −20° and was used for a maximum of 4 weeks after purification. Before every experiment, a suitable aliquot of the protein was removed, diluted 1:1 with reassembly buffer (0.1 M Mes, 1 mM EGTA, 0.5 mM $MgCl_2$, and 1 mM GTP, pH 6.4), and warmed to 37° for 20 min, and the microtubules were pelleted by centrifuging at 100,000*g* for 45 min at room temperature. In experiments to be done in the absence of Ca^{2+}, reassembly buffer was used to resuspend the pellet; in experiments to be done in the presence of Ca^{2+}, Mes–Ca^{2+} buffer (0.1 M Mes, 0.1 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 6.4) supplemented with 1 mM GTP and 1 mM $CaCl_2$ was used to resuspend the pellet.

Sedimentation velocity experiments were done using a Beckman Model E analytical ultracentrifuge and were recorded on metallographic plates (Kodak) with a schlieren optical system. The plates were read with a Nikon comparator. Schlieren peak areas were related to concentration by calibration with a bovine serum albumin standard using a synthetic boundary cell (Babul and Stellwagen, 1969). All experiments were done at a rotor speed of 47,660 rpm.

Microtubule protein was fractionated by resuspending the microtubule pellet to a final concentration of 10–20 mg/ml in Mes–Ca^{2+} buffer containing 1 mM $CaCl_2$ and 1 mM GTP, centrifuging out any fibrous precipitate at 500*g* for 5 min, and applying 1 ml of the supernatant to a 25 cm × 1.5 cm column containing Bio-Gel A-15M, 100–200 mesh (Bio-Rad). The protein was eluted with Mes–Ca^{2+} buffer.

Polyacrylamide–sodium dodecyl sulfate gel electrophoresis was carried out by the method of Laemmli (1970). Protein concentrations were determined by the Lowry *et al.* (1951) method, using bovine serum albumin as a standard.

Results

Purification of Microtubule Protein. By using two Beckman Type 21 rotors holding about 700 ml/rotor, it was generally possible to process six porcine brains in one purification, lasting a total of 15 hr. In a typical purification a homogenate of these brains contains about 7 g of soluble protein, which ultimately yields about 450 mg of purified microtubule protein. Sodium dodecyl sulfate gel electrophoresis of the product (Figure 1a) shows it to be about 95% pure, with the other 5% of the protein distributed in about 20 minor bands. Examined by electron microscopy, the isolated material has the characteristic appearance of microtubules (long protofilaments lying parallel to one another along the width of the tubule) (Kirschner and Williams, 1974). The product sediments as a heterogeneous boundary of >300 S except for a small amount of the protein which sediments as a 6S species.

Immediately before every experiment, the required amount of protein was repolymerized as described in the Experimental Section and pelleted at 100,000*g* for 45 min, and the supernatant discarded. The supernatant generally contained 20–30% of the total protein although this number went as high as 50% in preparations which had been stored for more than 3–4 weeks. This last contrifugation was in effect a third stage of purification which served to eliminate

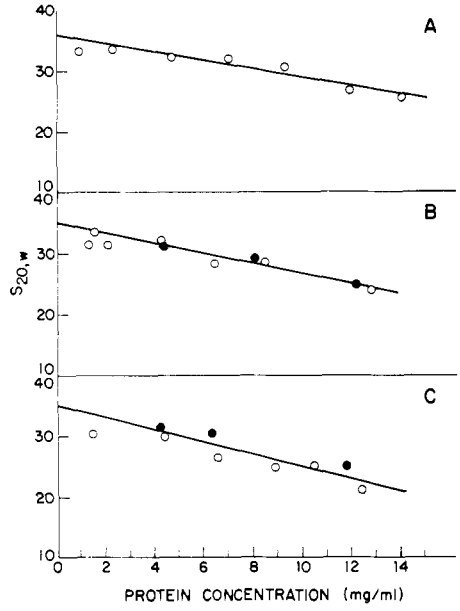

FIGURE 3: Concentration dependence of the sedimentation coefficient of the faster component. (A) Microtubule protein (15 mg/ml) in reassembly buffer was dialyzed overnight at 4° *vs.* reassembly buffer lacking GTP. Appropriate dilutions were made from the dialyzed protein and examined in the ultracentrifuge at 25°. (B) Microtubule protein was resuspended to a final concentration of 15 mg/ml in Mes-Ca^{2+} buffer containing 1 mM $CaCl_2$ and 1 mM GTP. The open circles represent appropriate dilutions of this protein examined in the ultracentrifuge at 25°. The filled circles represent samples which were incubated with 5×10^{-4} M colchicine for 45 min at room temperature before being analyzed in the same manner. (C) Microtubule protein was resuspended to a final concentration of 15 mg/ml in cold reassembly buffer containing 1 mM GTP. The open circles represent appropriate dilutions of this protein examined in the ultracentrifuge at 4°. The filled circles represent samples which were incubated with 5×10^{-4} M colchicine for 60 min at 4° before being analyzed in the same manner.

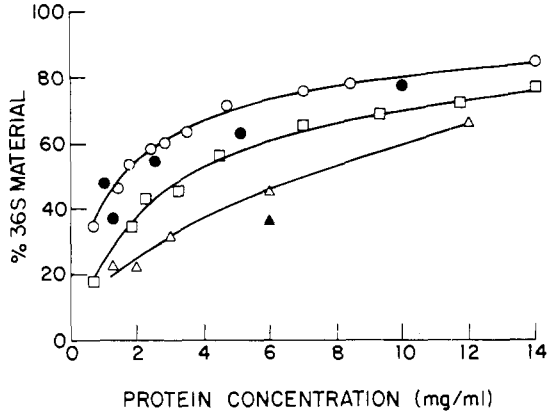

FIGURE 4: Dependence of the relative amount of the 36S component on total microtubule protein concentration. The amounts of the 6S and 36S components were determined from the areas under the schlieren peaks obtained in the ultracentrifuge: (○) microtubules depolymerized by addition of 2 mM $CaCl_2$ and analyzed at 25°; (●) Ca^{2+}-dissociated microtubules, incubated for 45 min at room temperature with 5×10^{-4} M colchicine before being analyzed; (□) microtubules dialyzed overnight at 4° to remove GTP, analyzed at 25°; (△) microtubules depolymerized by chilling to 0° for 30 min and analyzed at 4°; (▲) microtubules depolymerized by chilling to 0° for 60 min, incubated with 5×10^{-4} M colchicine at 0° for 60 min before being analyzed at 4°.

any protein which had lost the ability to polymerize during storage. Thus, in every experiment to be described, all of the protein used was derived from microtubules which had been reassembled immediately before the experiment began.

Two Major Components Present in Depolymerized Microtubules. The sedimentation pattern of the depolymerized brain microtubules is shown in Figure 2. Two components of about 6 and 30 S are seen regardless of whether depolymerization is effected by addition of 2 mM $CaCl_2$ (Figure 2c) or by chilling to 4° (Figure 2a). The same components are seen in the absence of $CaCl_2$ and at room temperature if the GTP in the sample is first removed by dialysis overnight to prevent repolymerization (Figure 2b).

Although all of the experiments described in this paper were done with porcine brain microtubules, the same isolation procedure has been used to purify microtubule protein from the brains of newly hatched chicks. Depolymerized microtubules from this source show the same sedimentation pattern as microtubules from porcine brain.

The slower component has the same sedimentation coefficient as the 6S colchicine binding protein studied earlier by Weisenberg *et al.* (1968). An extrapolation to infinite dilution of the sedimentation coefficient of the heavier component is shown in Figure 3. Under all of the depolymerizing conditions studied, the slope of the s *vs.* c plot is negative and extrapolates back to about 35 to 36 S at zero protein concentration. The s *vs.* c plot can be described by an equation of the form $s = s_0(1 - k_c)$, where k is a factor which depends largely on the size and asymmetry of the sedimenting species. The factor k for the 36S component is in the range 0.02–0.03 ml/mg which is consistent with it being a large and asymmetric structure (Creeth and Knight, 1965).

The relative amounts of the 6S and 36S components determined by analytical ultracentrifugation vary with protein concentration. As shown in Figure 4, the relative amount of the 36S component increases as the protein concentration is increased. At high protein concentrations, the Ca^{2+}-produced 36S component may form as much as 85% of the protein in the mixture, whereas at low concentrations the 36S component forms as little as 35% of the mixture. Although the exact composition varies from method to method, the same concentration dependence is seen qualitatively, independent of the procedure used for depolymerization. Since roughly 85% of the protein analyzed is recovered in the two components regardless of the protein concentration, the variability in the relative amounts of the two components cannot be due to preferential precipitation of the 6S component with increasing total protein concentration. It should be noted that the concentration of the 6S component does increase as the protein concentration is raised; hence, the increasing proportion of the 36S material in the mixture cannot be due to a concerted shift of the 6S protein into 36S oligomers above a critical concentration.

We have not made any attempt to correct the proportions of the 6S and 36S components in Figure 4 for the Johnston-Ogston effect (Johnston and Ogston, 1946) which, at high concentration, would tend to make the observed amount of 36S material less and the 6S material more than the true amount. An exact correction would require further physical parameters which are difficult to obtain experimentally. Nevertheless, the Johnston-Ogston effect is minimal when the two species under consideration differ sufficiently in their sedimentation coefficients. Making reasonable assumptions based on studies on similar systems (Schachman, 1959) we may estimate that the apparent percentage of 36S material in the mixture should differ from the true value by at most 10% at the highest concentrations examined. The

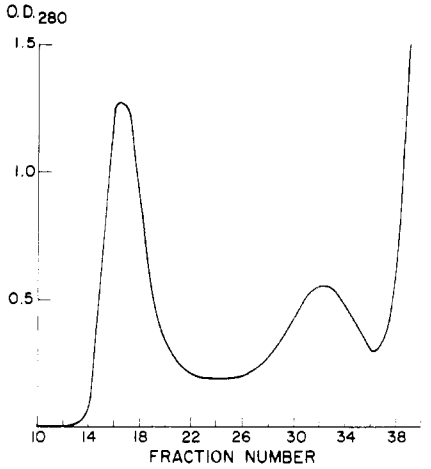

FIGURE 5: Agarose column fractionation of Ca^{2+}-depolymerized microtubules. Microtubule protein (1.5 ml of 10 mg/ml) was depolymerized by addition of 2 mM $CaCl_2$ and applied onto a 1.5 cm × 25 cm agarose 15m (100–200 mesh) column, equilibrated at 22° with Mes-Ca^{2+} buffer, and eluted with the same buffer, and 1-ml fractions were collected, at a flow rate of 15 ml/hr.

effect would be correspondingly less at lower concentrations.

We have found that the sedimentation pattern of the depolymerized microtubules does not change over a period of at least 10 hr at room temperature and at least 24 hr at 4°. In addition, the properties of the 6S and 36S components are independent of the number of polymerizations which the protein has undergone, as shown by the following experiment. A sample of microtubule protein was taken through six cycles of polymerization and depolymerization by alternately warming to 37° and chilling to 4°. The GTP concentration was kept constant at 1 mM throughout the experiment by dialysis, since previous experiments have shown that as little as two cycles of warming and cooling were sufficient to exhaust all of the GTP in the sample, thus preventing further polymerization. A sample treated in this manner was compared, by analytical ultracentrifugation, with a sample which had undergone only a single depolymerization. The two samples were identical with respect to the amounts of the 6S and 36S components and their sedimentation coefficients.

Effects of Colchicine and Divalent Cations. In Figures 3 and 4, microtubule protein samples containing 5×10^{-4} M colchicine are compared to control samples with respect to the relative amounts of the fast and slow components and their sedimentation coefficients. Both properties of the depolymerized mixture appear to be completely insensitive to colchicine. The only effect which has been noted is a broadening of the cold-produced 36S peak. Since no such broadening is seen at room temperature, it is possible that this is not due to a specific binding of colchicine to microtubule protein.

When the concentration of $MgCl_2$ or $CaCl_2$ is increased to 5 mM, a pronounced leading shoulder on the 36S component appears. At concentrations around 10 mM, a third peak appears, sedimenting as a heterodisperse species of approximately 71 S, which is formed at the expense of protein in the 6S and 36S peaks. It is likely that this represents large aggregates formed by association of protein from both the 6S and 36S components.

The complete absence of divalent cations, on the other hand, does not have any discernible effect on either of the two components. The complete absence of Ca^{2+} will, of course, lead to polymerization into microtubules. However, if the GTP in the sample is removed by dialysis prior to addition of EGTA to remove the Ca^{2+}, a normal 36S peak is seen in the analytical ultracentrifuge. Thus, Ca^{2+} ion is not required for the integrity of the 36S structure. Moreover, complete absence of any divalent cations, caused by overnight dialysis against 10 mM EDTA, does not have any noticeable effect on the amounts of the 6S and 36S components.

Fractionation of Depolymerized Microtubules. Due to the large size difference which exists between the 6S and 36S components, they can be separated by gel permeation chromatography on agarose (Bio-Gel A-15M) columns, as described in the Experimental Section. A typical elution profile is shown in Figure 5. The large increase in ultraviolet absorbance at the trailing end of the profile is due to GTP present in the original sample. Samples from the peak fractions were examined by analytical ultracentrifugation, to determine their purity. The leading fraction which elutes in the void volume is about 80% 36S material and 20% 6S, while the trailing fraction appears to be pure 6S protein with no detectable contamination by any of the 36S component.

In those cases where only purified 6S material was required, it could be obtained conveniently by centrifugation of $CaCl_2$-dissociated microtubules at 150,000*g* for 2.25 hr at room temperature. This was sufficient to pellet all of the 36S material, leaving only 6S protein in the supernatant, as determined by sedimentation analysis.

Sodium Dodecyl Sulfate Gels of 6S and 36S Proteins. Sodium dodecyl sulfate gel patterns of purified microtubule protein and of the isolated fractions are shown in Figure 1. At least 95% of the protein is present in two polypeptides (α and β tubulin) with molecular weights of 53,000 and 56,000, the rest being distributed in 15–20 minor bands. The same electrophoretic pattern has been previously demonstrated for isolated colchicine-binding protein by Olmsted *et al.* (1971), Bryan and Wilson (1971), and Feit *et al.* (1971). Isolated 6S and 36S fractions show precisely the same pattern, although the 36S fraction contains nearly all of the impurities which are visible on gels of whole microtubule protein. Even so, about 90% of the protein in the 36S fraction is contained in the α and β tubulin bands, as judged by densitometry of stained gels. In all cases, the α and β polypeptides are present in equal amounts.

Reversible Dissociation of the 36S Component into 6S Subunits. In view of the similarity in composition of the 6S and 36S fractions it was decided to investigate whether conditions existed which would break down the 36S component into a 6S subunit. It was found that (a) addition of 0.75 M NaCl, (b) raising the pH of the buffer to 10.5, or (c) addition of 0.1 mM *p*-hydroxymercuribenzoate or *N*-ethylmaleimide would eliminate the 36S peak with a concomitant increase in the amount of the 6S component. A typical result using 0.75 M NaCl is shown in Figure 6a,b.

Attempts to reassociate the 36S component by restoring the original conditions were successful only with the NaCl-dissociated material. In the case of dissociation by *p*-hydroxymercuribenzoate, addition of mercaptoethanol did not reverse the effect. Readjustment to pH 6.4 of a microtubule protein solution at pH 10.5 resulted in the formation of highly heterodisperse aggregates, possibly due to denaturation of the protein.

However, removal of the NaCl from salt-dissociated mi-

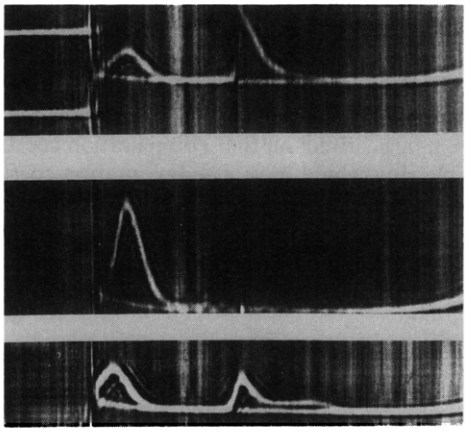

FIGURE 6: Dissociation and reassociation of the 36S component: (upper photograph) 7 mg/ml of microtubule protein in Mes-Ca^{2+} buffer containing 1 mM $CaCl_2$; (middle photograph) same as upper photograph, containing 0.75 M NaCl; (lower photograph) NaCl-dissociated sample, dialyzed for 3.5 hr *vs.* Mes-Ca^{2+} buffer + 1 mM $CaCl_2$ to remove the NaCl. All samples were analyzed at 23°, using a rotor speed of 47,660 rpm. The photographs were taken 9–14 min after reaching speed, using a schlieren phase plate setting at 75°.

crotubule protein resulted in the re-formation of 36S material indistinguishable from that present before salt dissociation (Figure 6c). Eighty-five per cent of the total protein and 67% of the 36S component present before salt dissociation are recovered after the NaCl is removed by dialysis for 3.5 hr at room temperature. This sample containing the reformed 36S material is fully competent to form microtubules, as judged by electron microscopy of a sample subjected to polymerizing conditions. Salt-dissociated microtubule protein which was centrifuged at 150,000g for 2.25 hr at room temperature was able to re-form as much 36S component as an uncentrifuged sample following removal of the NaCl. Since centrifugation removed virtually all of the impurities seen on sodium dodecyl sulfate gels, as well as any large microtubule protein aggregates, it is unlikely that either of these is necessary for 36S component re-formation. Furthermore, reappearance of the 36S component following dialysis is completely unaffected by the addition of 1 mM colchicine.

It should be noted that 6S protein purified from Ca^{2+}-depolymerized microtubules by high-speed centrifugation, treated with 0.75 M NaCl, and then restored to standard conditions by dialysis will not form any detectable amount of 36S material. Therefore, it seems that tubulin in the 6S form can associate into a 36S structure only when some of the 6S subunits were originally derived from the 36S component.

Molecular Weight of the 36S Component. The electron micrographs of Kirschner *et al.* (1974) reveal that the 36S component is in the form of spirals or double rings with outer diameters of 486 Å and inner diameters of 246 Å. The average total contour length of the two rings in the double ring structure is 2300 Å. Since it can be assumed that the particles in the inner ring must be in contact with those in the outer ring in order to maintain a rigid structure, we can assume that the width of each particle along the radius is 60 Å. These features of the double ring are presented in diagrammatic form in Figure 7.

The molecular weights of several hypothetical structures

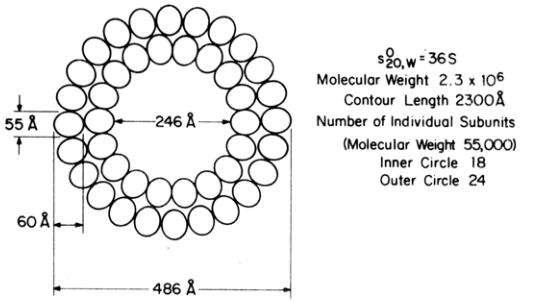

FIGURE 7: Model for microtubule double ring, deduced from electron microscopic data (Kirschner *et al.*, 1974) and sedimentation data.

of the fast sedimenting component, consistent with the electron microscope evidence, were calculated from the above dimensions. Knowing the sedimentation coefficient of the leading boundary to be 36 S, the molecular weights of these different structures can be calculated using the Svedberg equation, $M = sNf/1 - \bar{\nu}\rho$, where s is the sedimentation coefficient, f is the translational frictional coefficient, $\bar{\nu}$ is the partial specific volume of the particle, ρ is the solvent density, and N is Avogadro's number. The partial specific volume can be calculated from the amino acid composition and the densities of the amino acids (Cohn and Edsall, 1943). From the amino acid composition of α and β tubulin (Luduena and Woodward, 1973), we computed a $\bar{\nu}$ of 0.725 ml/g for microtubule protein. The solvent density of water was assumed to be unity. The frictional coefficients for various models of the double ring are calculated below.

The theoretical treatment of Kirkwood (1954) as has been employed by Bloomfield *et al.* (1967) allows for the determination of frictional coefficients of multisubunit structures by correcting for shielding of one subunit by another. If the subunits are of uniform shape and size, one can calculate the frictional coefficient of the entire structure using the equation

$$f = n\xi\left(1 + \frac{\xi}{6\pi\eta n}\sum_{i=1}^{n}\sum_{s=1}^{n}(R_{is}^{-1})\right)^{-1}$$

where n is the number of subunits, ξ is the frictional coefficient of one subunit, η is the viscosity of water, and $R_{i,s}$ is the center to center distance between any two subunits (taken twice between any two subunits due to reciprocal shielding from solvent).

The value for the sum of reciprocal distances in a double ring was calculated as follows: (1) inner–inner and outer–outer interactions (derived from the equation for the length of a chord)

$$\Sigma(R)^{-1} = \frac{n}{2}\sum_{a=1}^{n-1}(2r \sin(\Pi a/n))^{-1}$$

where n is the number of subunits in either the inner or outer ring and r is the radius to the center of a particle in either the inner or outer ring; (2) inner–outer interactions (derived from the cosine law)

$$\Sigma(R)^{-1} = n_o\sum_{a=1}^{n_i}(r_i^2 + r_o^2 - (r_o)(r_i)\cos(2\Pi a/n_i))^{-1/2}$$

where n_i and n_o are the numbers of particles and r_i and r_o are the radii of the inner and outer rings, respectively.

The above calculation was performed for three different models of the double ring structure in order to test the accuracy of this approach. In the first calculation, the equation was applied to a structure composed of 22 outer and 16

inner spheres of 60-Å diameter (38 such structures give a good fit for the mean contour length). The subunit frictional coefficient derived by the Stokes equation was 5.65×10^{-8} g/sec, and the total frictional coefficient calculated for this model was 2.99×10^{-7} g/sec. With an $s_{20,w}{}^0$ of 36 S, this structure would have a molecular weight of 2.36×10^6.

In order to test the sensitivity of this treatment to the fact that the subunits are not spherical, this calculation was also performed on a model of 76 ellipsoidal units (n_i 32; n_o = 44) with a width along the radius of 60 Å. Each subunit in this structure would be spaced every 30 Å along the ring to give a total contour length of 2300 Å. If it is assumed that the molecular weight of each subunit is 55,000 daltons, the thickness can be estimated from the known anhydrous volume ($M\bar{v}/N$) of such a molecule to be 40 Å. Assuming that the subunit can be approximated by an ellipsoid of revolution, its frictional coefficient can be calculated as follows

$$\xi = (f/f_o)(6\Pi\eta r_o)$$

where f/f_o is the correction for asymmetry of a particle whose equivalant spherical volume has a radius of r_o. For an ellipsoid of axes, *a, b,* and *c*, $r_o = (a \times b \times c)^{1/3}$. In this case the axial ratio is about 2 and $f/f_o = 1.044$ (Svedberg and Pedersen, 1940). Knowing ξ and the sum of $(R)^{-1}$, the frictional coefficient for a double ring of 76 ellipsoidal subunits was found to be 2.85×10^{-7} g/sec, which corresponds to a molecular weight of 2.25×10^6.

Therefore, doubling the number of subunits within the confines of the same planar dimensions for the double ring resulted in less than a 5% change in the frictional coefficient and hence in the molecular weight of the structure. The molecular weight thus calculated for either model is about 2.3×10^6 and corresponds to a molecule composed of 42 polypeptides of 55,000 daltons each.

We can now recalculate the frictional coefficient for a model of the double ring with 42 subunits, 18 on the inner ring and 24 on the outer ring. The individual subunits in this model are spaced every 55 Å along the rings and are therefore estimated to be 21 Å thick. The 55,000-dalton subunit would then be approximated as an oblate elipsoid with an axial ratio of 3. The calculated frictional coefficient for this double ring structure is 2.88×10^{-7} g/sec, yielding a value of 2.27×10^6 for the molecular weight. It is therefore seen that a significant change in the thickness of the ring has negligible effect on its frictional coefficient and hence on the estimate for the molecular weight. This model for the double ring containing 42 subunits of molecular weight 55,000 is shown in Figure 7.

Electron micrographs indicate that under specific conditions the fast sedimenting component may be found as spirals, as double rings, as double rings with additional subunits on the outside, sometimes even approaching triple rings (Kirschner and Williams, 1974), or as single rings (G. G. Borisy and J. B. Olmsted, personal communication). It is therefore important to determine what the sedimentation properties of such structures would be so that they may be recognized. We have applied the Kirkwood calculation to individual rings of the dimensions of the outer and inner rings of the double ring structure. The sedimentation coefficient of the inner ring of the molecular weight 0.99×10^6 would be 21 S and that of the outer ring, molecular weight 1.32×10^6, would be 22 S. Therefore, a variety of rings of approximately these dimensions would have similar sedimentation coefficients of approximately 21 S, as would be expected for open free draining structures with little shielding.

The calculation was also applied to a triple ring model, with an outer diameter of 620 Å. Increasing the number of subunits around a double ring to form a triple ring is found to have a relatively small effect on the frictional coefficient and hence a large effect on the sedimentation coefficient, so that a triple ring would have a sedimentation coefficient of 52 S. Such a structure may in fact be observed in calcium-dissociated microtubule protein, which, as has been mentioned, has a heavy heterogeneous shoulder on the 36S boundary.

Discussion

The results presented here indicate that brain microtubules depolymerize to form two components, one with a sedimentation coefficient of 6 S and the other with a sedimentation coefficient of 36 S. Since the same two distinct structures are formed under a number of different depolymerizing conditions from microtubules isolated from two different species, they appear to be significant components of microtubules rather than fortuitous breakdown products. The 6S component is identical with the previously isolated colchicine binding protein in terms of its polypeptide chain composition, its sedimentation coefficient, and its colchicine binding properties. The 36S component probably represents the "disks" which have been observed earlier under the electron microscope (Borisy and Olmsted, 1972). This correlation is strengthened by the disappearance of the 36S sedimentation boundary, as well as "disks" under polymerizing conditions (Kirschner *et al.*, 1974).

The achievement of a partial separation of the 36S and 6S material by gel permeation chromatography has enabled us to study the properties and interactions of these two components. The trailing fraction from the agarose column, composed solely of 6S tubulin, and the leading fraction composed of mostly 36S tubulin show the same two α and β polypeptide bands on sodium dodecyl sulfate-acrylamide gel electrophoresis. In addition, the 36S component appears to be made up of 6S subunits, since addition of 0.75 M NaCl to the entire depolymerization mixture causes a complete breakdown of the 36S boundary and a corresponding increase in the amount of material in the 6S boundary.

Since neither the leading nor trailing component contains any major polypeptide bands on sodium dodecyl sulfate-gels other than α and β tubulin, it is likely that no other major structural proteins are required for polymerization. Borisy *et al.* (1974) have found a high molecular weight polypeptide, besides α and β tubulin, which in their preparations forms about 15% of the protein and which appears to copurify with microtubule protein. It is not clear whether this component has an affinity for microtubules or is merely a contaminant which also has the property of polymerizing in the warm and depolymerizing in the cold under the purification conditions used by Borisy. Since our preparations lack this protein and still polymerize readily, it is unlikely that this high molecular weight material is required for polymerization. The breakdown of the 36S material by NaCl and re-formation in good yield by removal of NaCl suggest that all of the information necessary to form the 36S structure must be present in the purified preparation of microtubule protein. No other activity in crude brain extracts therefore seems to be required for assembly of this structure.

It has been reported by Weisenberg and Timasheff

(1970) that 6S tubulin assayed by its colchicine binding capacity and purified by DEAE-Sephadex chromatography can form a 30S aggregate under certain conditions. The relationship of this 30S component to our 36S component is unclear, especially since the colchicine binding protein isolated by this method does not polymerize into microtubules. The 30S boundary required high concentrations of divalent cations in order to form and its formation was enhanced by colchicine. Neither of these properties is true of the 36S component obtained from depolymerized microtubules. Furthermore, the 30S peak bound more colchicine by weight than did the 6S tubulin, while the reverse is true of our 36S component (Kirschner *et al.*, 1974). Nevertheless, the results of Weisenberg and Timasheff (1970) indicate that conditions exist under which 6S tubulin has a tendency to aggregate into a 30S form, and it is possible that a modification of this protein, as suggested below, could enhance this tendency and allow the formation of a 36S aggregate under conditions not requiring divalent cations or colchicine.

Although the 36S material has been referred to as disks, recent high-resolution electron microscopy of material from purified preparations of microtubule protein has shown that it exists in two main forms, double rings and spirals (Kirschner *et al.*, 1974). The structure of the double ring, deduced by combining electron microscopic data and sedimentation data, and shown schematically in Figure 7, deserves some discussion. For a number of reasons, the double ring does not appear to be derived from a microtubule cross section. First there is no evidence that microtubules are composed of double layered concentric cylinders. Second, the outside diameter of a microtubule is 250 Å while the diameter of the inner ring of the 36S structure is 360 Å. Finally, the inner ring of the 36S structure contains 18 subunits while microtubules are composed of an array of only 13 protofilaments (Olmsted and Borisy, 1973).

Another interesting feature of the double ring is that the number of subunits in the outer ring cannot be an integral multiple of the number in the inner ring. This precludes the possibility that each subunit in the inner ring bonds to a subunit in the outer ring in the same steric relationship. This is especially surprising because in the microtubule, adjacent protofilaments (from which it is thought that double rings and spirals arise (Kirschner *et al.*, 1974)) are parallel and the subunits can be in a one-to-one correspondence. In other biological assembly systems these regular bonding relationships are provided by helical twists or multilayering, both of which appear to be absent in this structure. The difficulty in explaining how the inner and outer rings are held together can be overcome in a number of ways. (a) A large number of protein–protein binding sites could be present on each subunit, which would allow staggering of one subunit relative to another and still allow bond formation between all subunits on the inner and outer rings. (b) There may be only a few subunits in the inner ring which bond to correctly positioned subunits in the outer ring. For example, in the model shown in Figure 7 every third subunit in the inner ring could bind to every fourth subunit in the outer ring. All the other subunits would be held in position by longitudinal bonding within the ring they occupy. (c) Some bonding mechanism other than the presence of specific binding sites, *e.g.*, electrostatic interaction, could be operative. The size of the double ring which is quite homogeneous could be dictated by a requirement for a certain curvature in one or both rings. The inner ring, for example, may be a minimum size for positioning of one subunit relative to another around the ring. Increased mismatching could also limit the maximum size of spirals. In any case microtubule double rings and spirals pose interesting structural questions which may not have been encountered previously in other systems such as virus assembly.

As the protein concentration is increased the proportion of material in the faster sedimenting boundary increases and this suggests that the 6S and 36S components are involved in some sort of equilibrium process. In general there are two types of equilibrium processes that one could envisage. In the first there is simply a monomer–polymer equilibrium, where increasing the total concentration causes the production of a greater number of polymer molecules, whose size remains constant. In the second there are a fixed number of polymeric nucleating centers and increasing the concentration increases the size of the polymer species, but their number remains constant. These two modes could suggest quite different mechanisms for microtubule assembly. Especially with respect to the first type of equilibrium, it should be borne in mind that 6S tubulin isolated either on agarose columns or by high-speed centrifugation shows no tendency to form any 36S materials. Therefore, if the first equilibrium process applies to the protein being studied here, it would imply that two types of 6S subunits must exist, only one of which is in equilibrium with 36S structures.

In both cases an increase in concentration would cause an increase in the proportion of material in the polymer fraction, but in the second case the molecular weight would increase and it is expected that the sedimentation coefficient would increase as well. However, depending on the structure of the polymeric species an increase in molecular weight could cause only a small increase in sedimentation coefficient if there were a large increase in frictional coefficient. Under these circumstances the increase in sedimentation coefficient could be more than compensated for by the increasing viscosity and backward flow effects in the ultracentrifuge (Smith *et al.*, 1973), leading to a consistently negative slope for a plot of s *vs.* c. In the absence of other information the observed decrease in sedimentation coefficient of the faster boundary with concentration (Figure 3) could only be used effectively to support the first model if one could estimate the hydrodynamic effects of increased concentration. Unfortunately no satisfactory theory exists for such an estimate (Schachman, 1959; Smith *et al.*, 1973).

Knowledge of the structure of the 36S particle, such as obtained from electron microscopy, could be used to distinguish between these two models. If the increase in the proportion of 36S material from 45% at 2 mg/ml to 82% at 10 mg/ml were due to addition of subunits to the double ring structure to form a triple ring or to form a double thickness double ring, there would be a relatively small increase in the frictional coefficient and therefore a substantial increase in sedimentation coefficient from 36 to 52 S in the former case and from 36 to 69 S in the latter case. Such a steep increase could not in all likelihood be compensated for by hydrodynamic effects to give the observed decrease of 33–27 S. Therefore, we must conclude under these assumptions of how subunits would add to double rings that the shift in the equilibrium with concentration is due in part at least to an increase in the number of double rings. Electron microscopic evidence supports this view since size of the double rings is homogeneous and few multiple ring structures are seen.

The presence of two interacting components so similar in composition could be explained by a number of hypotheses which are outlined and discussed below. (a) The 36S component is formed by irreversible aggregation of the 6S protein, possibly as a result of denaturation. (b) The 36S component is the functional form of microtubule protein, and the 6S subunits observed in the ultracentrifuge are denatured forms of the protein. (c) Only a single microtubule protein exists, and it is distributed in an equilibrium fashion between the 6S and 36S forms. (d) The protein which forms the 36S component is distinct from the protein occurring in the 6S form, *i.e.*, they are coded for by separate genes. Since the two proteins are similar, they would most likely be related in an evolutionary sense, much like the α and β chains of hemoglobin. (e) A cofactor present in limiting amounts binds to some 6S subunits, enabling them to aggregate into a 36S structure. (f) Some of the 6S tubulin subunits are modified, *e.g.*, phosphorylated or acetylated, in order to convert them to a form which will associate into a 36S structure.

In models e and f, the converse is, of course, also possible, *i.e.*, modification or binding of a cofactor could be required for formation of the 6S component.

Insofar as reincorporation into microtubules is an indication of nativeness, neither component can be a denaturation product of the other component, as implied in models a and b. All of the 36S material and 65% of the 6S material are reincorporated into microtubules under polymerizing conditions (Kirschner *et al.*, 1974). Moreover, half of the remaining 35% of the 6S component will enter microtubules when mixed with fresh microtubule protein, as experiments with radioactively labeled protein have shown (D. R. Littman and M. W. Kirschner, manuscript in preparation). Also, if either component were a denaturation product, it would be expected to increase in amount either as a function of time or as a function of the number of times a sample has been polymerized. In fact, there is no dependence on either of these factors.

Models which suggest that the 6S and 36S components are derived from precisely the same protein (models a and c) ignore two important observations. First, the 6S component can be isolated in pure form and shows no tendency to form any 36S material. Secondly, the two components differ greatly in their ability to bind colchicine (Kirschner *et al.*, 1974), even when both are reduced to a 6S form by addition of 1 M NaCl.

Models d, e, and f remain as the most plausible alternatives. Although it is not yet possible to determine which one is correct, implicit in all three models is the same general feature, namely that two types of tubulin exist. One form, which has been previously referred to as X-tubulin, would occur only as the free 6S subunit, and is found in the trailing fraction of the agarose column. The other form (Y-tubulin) would occur primarily as the 36S aggregate, and is found in the leading fraction of the agarose column. With reference to model f, phosphorylation of microtubule protein is known to occur (Eipper, 1974). We have recently found that nearly all of the hot Cl_3CCOOH-precipitable phosphate in microtubule protein which has been fractionated on agarose columns occurs in the leading fraction, which contains the 36S component (M. W. Kirschner and M. M. Suter, unpublished observations). Modification of microtubule protein by phosphorylation is therefore considered by us to be a likely mechanism by which X- and Y-tubulin could be formed.

One of the major differences between X- and Y-tubulin is in their interaction with colchicine, which completely inhibits microtubule polymerization. X-tubulin binds colchicine readily; Y-tubulin binds colchicine poorly (Kirschner *et al.*, 1974). Neither the sedimentation coefficient nor the amount of the 36S component is affected by colchicine, as shown in Figures 3 and 4, nor is re-formation of the 36S component from salt-dissociated microtubule protein affected by the presence of high concentrations of this drug. These facts suggest that colchicine does not bind appreciably to, and has little effect on, the 36S component under depolymerizing conditions, implying that it may prevent microtubule polymerization by interfering with the 6S component. The 6S subunits which have bound colchicine may be incapable of allowing microtubule protofilaments to assume their normal arrangement, leading to the abortive polymerization products which have been observed earlier in the presence of colchicine (Kirschner and Williams, 1974).

The presence of two types of tubulin could offer significant biological advantages *in vivo*: First, a cell could regulate the number of microtubules formed under polymerizing conditions by regulating the conversion of X-tubulin to Y-tubulin, since X-tubulin by itself does not polymerize (Kirschner *et al.*, 1974). It follows from this that a cell could synthesize large amounts of tubulin, and in effect hold them in reserve until synthesis of more microtubules was required. Second, the presence of pre-formed 36S "packages," corresponding to protofilament lengths of 0.23 μ, would probably increase the speed with which microtubules could be polymerized, compared to a system in which assembly was directly from 6S subunits. Finally, it is possible that varying the relative amounts of the two components would result in variations in the nature of the microtubules formed; for example, the flexibility of a microtubule could vary depending on the relative amounts of X- and Y-tubulin which it contained. It is clear, in any case, that detailed elucidation of the differences between X-tubulin and Y-tubulin *in vitro* would very likely lead to a better understanding of how microtubule assembly is controlled *in vivo*.

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Equilibrium Binding of Estradiol by Uterine Cell Suspensions and Whole Uteri *in Vitro*†

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ABSTRACT: The nature of estradiol binding to specific uterine estradiol binding proteins was studied in the intact cell at a temperature of 37°. Titration of the estradiol binding sites in uterine cell suspensions showed no cooperative behavior with equilibrium estradiol concentrations providing 5% to over 95% saturation of the binding protein. Hill plots of these data yielded a slope of 1.03 ± 0.06 (SEM). Binding studies on whole uterus incubations *in vitro* produced similar results. Hill plots of these data yielded a slope of 1.06 ± 0.05 (SEM). These results led us to conclude that estradiol binding under these conditions occurred as a simple equilibrium with independent binding sites.

A variety of studies have examined the properties of the uterine estradiol binding proteins. Several reports have suggested the presence of cooperative behavior in estradiol binding to proteins present in cell-free extracts at 0° (Ellis and Ringold, 1971; Puca *et al.,* 1971; Sanborn *et al.,* 1971). On the other hand, numerous studies have shown no evidence of cooperative behavior under similar experimental conditions (Clark and Gorski, 1969; Giannopoulos and Gorski, 1971; Notides, 1970). Because of the conflicting nature of these reports, we examined the process of estradiol binding by these proteins as it occurs within the environment of the intact cell at physiological temperatures. The nature of the binding process under these conditions is also of interest in view of studies showing that an *in vitro* uterine response to estradiol is directly proportional to the quantity of the estradiol binding protein complex present in that tissue (Katzenellenbogen and Gorski, 1972). The experiments reported here concern equilibrium binding of estradiol by whole uteri and cell suspensions prepared from these uteri.

Materials and Methods

Free Cell Suspensions. The preparation of cell suspensions from immature rat uterus, conditions of incubation, and procedures for determination of specifically bound estradiol were exactly as described previously (Williams and Gorski, 1973). Briefly, the suspensions were prepared from uteri of 20- to 24-day-old Holtzman rats. Collagenase and Pronase were used in the second stage of the dissociation procedure. The cells were suspended in Eagle's HeLa medium containing 0.1% methyl cellulose under an atmosphere of 95% O_2–5% CO_2 (pH 7.4, 37°) and incubated with [^{3}H]estradiol (17β-[6,7-^{3}H]estradiol, 40 Ci/mmol, New England Nuclear) or [^{3}H]estradiol and excess unlabeled estradiol (Mann Research) for 70 min at 37°. The incubations with the hormone were initiated by diluting the samples with equal volumes of medium containing various hormone concentrations, or by diluting the samples with varying volumes of medium at various hormone concentrations. In the former case the final cell concentration was 0.5 × 10^7/ml while the latter procedure yielded a cell concentra-

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