# COMPARISON OF THE SEDIMENTATION PROPERTIES OF MICROTUBULE PROTEIN OLIGOMERS PREPARED BY TWO DIFFERENT PROCEDURES

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SUMMARY: Previously reported values of 30S and 36S for the sedimentation coefficients of microtubule protein oligomers prepared by two different methods (Borisy et al., Annals N.Y. Acad. Sci. 253, 107-132 [1975]; Weingarten et al., Biochemistry 13, 5529-5537 [1974]) and analysed under different conditions have been confirmed. These differences in sedimentation coefficients were also observed when the two classes of oligomers were analysed under the same conditions, both at 5°C in the absence of $Ca^{++}$ and at 24.5°C in the presence of $Ca^{++}$. Gel electrophoresis showed differences in composition, the principal difference being a reduced amount of some high molecular weight (ca. 3 x $10^5$ Daltons) proteins in the samples prepared by the Weingarten et al. method. We conclude that the two procedures produce genuine differences in the properties of microtubule protein.

Several groups have now reported that ring-shaped tubulin oligomers are formed as products of the depolymerization of mammalian brain microtubules in vitro (1-5). The structure of the tubulin oligomer has been of some interest because of the possibility that the ring oligomer serves as an intermediate in the pathway of microtubule self-assembly. However, the properties of these oligomers and their role in the mechanism of microtubule polymerization are in dispute. Kirschner's group has characterized the tubulin oligomer as a 36S species by sedimentation velocity centrifugation and as a double-ring structure by electron microscopy (6,7); whereas we have described two species of oligomers with sedimentation coefficients of 20S and 30S, respectively, and have observed only a single-ring structure by electron microscopy (2,8).

The reported differences in ring structures have not been uniformly appreciated, and the various tubulin oligomers have been referred to in the literature as essentially the same structure and as "30-36S rings" (7,9,10). Therefore we have undertaken the current study to evaluate the reported differences between our work and that of Kirschner's group and to determine whether the reported differences are due to intrinsic properties of the oligomers or are attributable to the conditions of investigation.

## Materials and Methods

Protein purification. Microtubule protein was prepared from porcine brain tissue by two different methods, both involving successive cycles of temperature-

mediated polymerization and depolymerization with alternate cycles of centrifugation to purify the samples. The first perparative method, which we shall call the standard method since it is generally used in this laboratory, was that of Borisy et al. (8). For this procedure only the cerebral cortex was used. Tissue was homogenized in PMG (0.1 M PIPES*, 0.1 mM $MgCl_2$, and 1.0 mM GTP at pH 6.94). Samples were used after three cycles of polymerization at 37°C for 30 minutes and depolymerization at 0°C for 30 minutes alternated with 39,000 x g centrifugation steps, at 37°C for 40 minutes to pellet polymerized microtubules, or at 0°C for 30 minutes to clarify the depolymerized samples. The second method was that of Shelanski et al. (11) as adapted by Weingarten et al. (7) in which whole brains were stripped of meninges and homogenized in MEMG (0.1 M MES*, 1.0 mM EGTA, 0.5 mM $MgCl_2$, and 1.0 mM GTP at pH 6.4) with 1.0 mM EDTA and 1.0 mM 2-mercaptoethanol added. This buffer was used for the 0°C depolymerization steps, and the same buffer containing 4 M glycerol was used for the 37°C polymerization steps. Purified, sedimented microtubules were centrifugally washed to remove excess glycerol by a cycle of depolymerization-polymerization in experimental buffer. For this procedure protein was resuspended in experimental buffer, depolymerized at 0°C for 30 minutes and centrifuged at 39,000 x g and 4°C for 30 minutes; the supernatant was polymerized at 37°C for 30 minutes and centrifuged at 39,000 x g for 40 minutes to collect the washed microtubules. Samples were then resuspended and depolymerized at 0°C for 30 minutes and diluted to the desired concentrations before analytical centrifugation.

Analytical centrifugation of tubulin oligomers was performed by the sedimentation velocity method in a Spinco Model E centrifuge at a rotor speed of 42,040 rpm, using a titanium AN-F (4-cell) rotor and 12 mm double-sector cells with either Kel-F or charcoal-filled Epon centerpieces. Photographs were made with the Schlieren optical system on Kodak Contrast Process Ortho film. Sedimentation coefficients were calculated by standard procedures after measurement of the distances of the peaks from the rotor reference hole on a Gaertner microcomparator.

Gel electrophoresis was carried out by the method of Shapiro et al. (12), with 3.7% acrylamide gels and staining on 0.025% Coomassie Blue in 10:25:65 acetic acid:isopropanol:water (v:v:v) after Fairbanks et al. (13).

Protein concentrations were determined by the method of Lowry et al. (14), with bovine serum albumin as the standard.

## Results and Discussion

Sedimentation analysis. In both Kirschner's studies (3,6,7) and our own (1,2,8) porcine brain was the source tissue and tubulin oligomers were obtained as depolymerization products from microtubules in vitro; therefore, neither source tissue nor method of obtaining oligomers differed in the two studies. However, the two studies did differ in (a) the procedures for purifying the microtubule protein and (b) the solution conditions for analysing the oligomers. Therefore, these differences were examined as possible origins of the reported differences in oligomer properties.

Protein prepared by our standard method, in the absence of glycerol, was depolymerized either by resuspension in the preparative buffer (PMG) at 0°C or

* Abbreviations: PIPES, monosodium piperazine-N,N'-bis-(2-ethane sulfonate)
MES, sodium morpholinoethanesulfonate

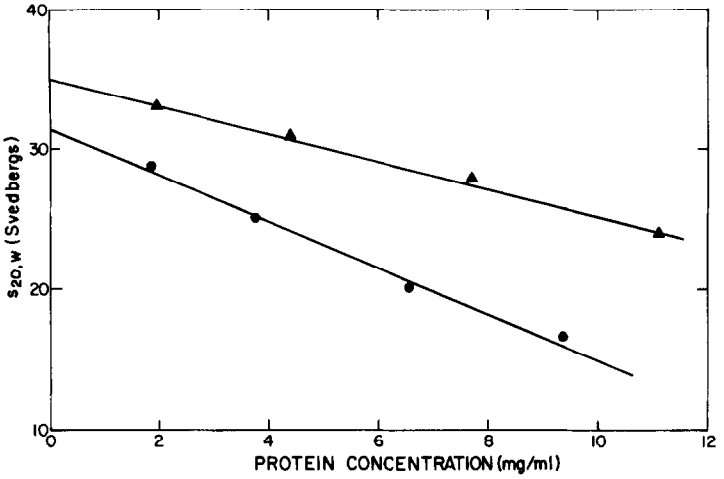


Figure 1. Sedimentation coefficients of tubulin oligomers at 5°C, corrected to $s_{20,w}$, as functions of total protein concentrations; ▲, glycerol-prepared sample in 0.1 M MES, 1.0 mM EGTA, 0.5 mM $MgCl_2$, 1.0 mM GTP, pH 6.4; ●, standard preparation in 0.1 M PIPES, 0.1 mM $MgCl_2$, 1.0 mM GTP, pH 6.94. Rotor speed was 42,040 rpm. The line for the glycerol-prepared sample coincides with the line of Figure 3c of Weingarten et al. (7) for the same type of preparation at 4°C.

by resuspension at room temperature in the same buffer containing 2.0 mM $CaCl_2$. Examination of such samples in the analytical centrifuge showed peaks with extrapolated sedimentation coefficients ($s^{\circ}_{20,w}$) of 5.8S, ca. 20S and 31±1S at 5°C in the absence of $Ca^{++}$. In the presence of $Ca^{++}$ at 24.5°C, the fastest sedimenting species had a slightly increased (8±2%) sedimentation coefficient, as previously reported (2). Polymerized, glycerol-prepared protein was readied for analytical centrifugation under the conditions described by Weingarten et al. (7) except that, to remove glycerol, the sample was centrifugally washed in the experimental buffer instead of being dialysed. The experimental buffer was MEMG (0.1 M MES, 1.0 mM EGTA, 0.5 mM $MgCl_2$ and 1.0 mM GTP at pH 6.4). In some experiments 2.0 mM $CaCl_2$ was added. The depolymerized microtubule protein showed two peaks, the dimer peak at 5.8S and an oligomer peak at 36±1S. In the presence of $Ca^{++}$ at 24.5°C the faster species again sedimented at 36S. The concentration dependence of the sedimentation coefficients and the extrapolation of the coefficients to infinite dilution for experiments at 5°C in the absence of $Ca^{++}$ are shown in Figure 1. It is seen that at all protein concentrations the sedimentation coefficient of the glycerol-tubulin oligomer was greater than that of the standard tubulin oligomer. These differences in sedimentation coefficients were observed for oligomers derived from both cold-depolymerized and $Ca^{++}$- depolymerized protein. The agreement of our data with those of Kirschner's group is shown by

Table I.

Sedimentation coefficients of microtubule protein oligomers

| T (°C) | Buffer | Standard preparation[a] | | Glycerol preparation[b] | |
|---|---|---|---|---|---|
| | | $s^{\circ}_{20,w}$ (Svedbergs)[c] | | | |
| 5.0 | PMG[d] | (20) | 31.5 | 21.1 | - |
| | MEMG[e] | - | 31.5 | - | 35.1 |
| 24.5 | PMG +2 mM $Ca^{++}$ | (20) | 33.3 | 20.7 | 37.6 |
| | MEMG +2 mM $Ca^{++}$ | - | 31.2 | - | 35.8 |

[a]Protein prepared according to the method of Borisy et al. (8).

[b]Protein prepared according to the method of Shelanski et al. (11) as adapted by Weingarten et al. (7).

[c]Values of $s^{\circ}_{20,w}$ were obtained for each condition by measurements of $s_{20,w}$ over a range of concentrations and by extrapolation to infinite dilution.

[d]PMG: 0.1 M PIPES, 0.1 mM $MgCl_2$, 1.0 mM GTP at pH 6.94.

[e]MEMG: 0.1 M MES, 1.0 mM EGTA, 0.5 mM $MgCl_2$, 1.0 mM GTP at pH 6.4.

Figures in parentheses indicate that the 20S oligomer was present but not at high enough concentrations to extrapolate the sedimentation coefficient curve accurately to infinite dilution. Blanks denote that under the conditions indicated the corresponding oligomer was not detected.

the fact that the line drawn through the glycerol-tubulin points in Figure 1 coincides with the line in Figure 3c of Weingarten et al. (7). Thus the results of Weingarten et al. (7) have been substantially reproduced, confirming the reported differences in the sedimentation properties of the two classes of oligomers.

We then undertook to determine whether the differences in sedimentation coefficients were due to the differences in the solution conditions used for the analytical centrifugation studies or to inherent differences in the oligomers derived by the two preparative procedures. Samples of each oligomer type were transferred to the solutions previously used to analyse the other type by an extra cycle of polymerization-depolymerization with centrifugal washing in the appropriate solution. For each solution condition sedimentation coefficients were measured over a range of protein concentration, and values of $s^{\circ}_{20,w}$ were determined by extrapolation to infinite dilution. As shown in Table I, the

standard protein at 5°C in MEMG buffer at pH 6.4 showed only the 6S and 30S species, behaving normally and not like the glycerol-prepared protein. In the presence of 2 mM $CaCl_2$ in MEMG buffer at 24.5°C the standard protein again showed only the 6S and 30S peaks. Hence the standard tubulin oligomer, when analysed under the solution conditions employed by Weingarten et al. (7), retained its sedimentation coefficient of 31S and did not take on the value of 36S. Only a reduction in the amount of the 20S species was caused by the change in solution conditions. A description of the results for the analysis of glycerol-tubulin oligomers under the conditions previously used for the standard protein is somewhat more complicated. In the absence of $Ca^{++}$ the glycerol-tubulin oligomer was converted to 21S material upon transfer to PMG at pH 6.94 so that determination of the sedimentation coefficient of the 36S component under these conditions was not possible. When glycerol-tubulin preparations were transferred to PMG at pH 6.94 with 2 mM $CaCl_2$ the principal peak still sedimented at ca. 36S, although some new 21S material was also produced.

From the above results we conclude that the reported differences in sedimentation coefficients of the 31S and 36S oligomers of microtubule protein are reproducible and are inherent differences resulting from differences in the preparative procedures rather than from differences in the conditions of investigation. In addition, the increased concentration of 20S material in the higher pH solution is consistent with the known pH dependence of the relative abundance of the oligomer species present in the standard preparations. It has been shown that at any given ionic strength the concentration of 20S material is a smooth function of pH in 50 mM PIPES buffer, disappearing completely at pH < 6.5 at ionic strengths up to 0.28 (NaCl augmented) for 5 mg/ml standard protein at 5°C (Marcum, J.M. and Borisy, G.G., manuscript in preparation).

Electrophoretic analysis. Previously we have characterized our preparations of microtubule protein as consisting of 75% tubulin and 15-20% high molecular weight (HMW) proteins (ca. $3 \times 10^5$ Daltons) and the rest in several minor bands distributed throughout the gel (8). However Weingarten et al. (7) have reported their protein to be 95% tubulin and to contain no high molecular weight species. Therefore, in consideration of the possibility that the differences in composition might be a reason for inherent differences in the sedimentation properties of the two systems, we have assessed the reproducibility of the reported compositional differences. We have analysed samples of both types of microtubule protein prepared and purified exactly as they were for the analytical centrifugation experiments. Electrophoretic gels with identical loads of samples of the two kinds of preparation were run together. Results showed the standard protein to have approximately the composition previously reported (8), 74±4% tubulin, and 23±1% HMW proteins (see Figure 2, top trace). In contrast, in the glycerol prep-

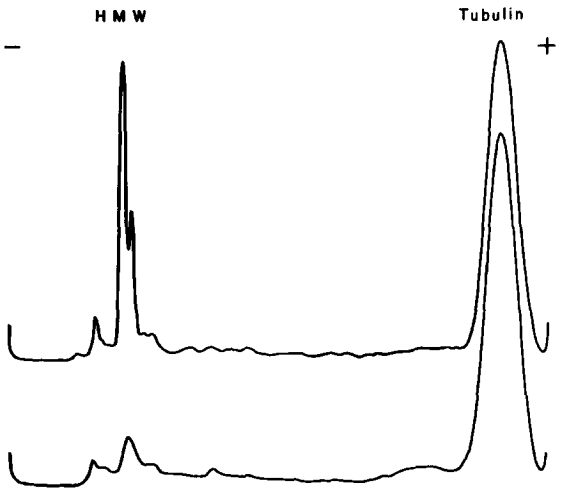


Figure 2. Densitometer tracings of 3.7% polyacrylamide gel electrophoretic patterns, stained with Coomassie Blue. Upper trace, standard preparation after 3 cycles of purification. Lower trace, glycerol preparation at the same stage of purification. Both gels were loaded with a total of 10 µg of protein in a volume of 100 µl.

arations 84±1% of the total stain on the gel was in the tubulin band and 9±1% in the HMW region. Thus here, as in the analytical centrifugation experiments, differences between the two types of preparations were evident. However, our observations on the composition of the glycerol-isolated tubulin differed somewhat from the results of Weingarten et al. (7) since we did observe some high molecular species to be present.

The confirmation of the differences in sedimentation properties of the oligomers convinces us that the two preparative procedures produce genuine differences in the behaviour of the protein. Whether these differences should be attributed to compositional differences or to some other factor is not entirely clear. In this regard, however, it should be noted that Detrich et al. (15) have shown that microtubule protein prepared by a glycerol procedure has glycerol bound to both the 6S fraction and to the oligomer fraction. A portion of the bound glycerol was not removable by gel filtration or by prolonged dialysis and was therefore apparently non-exchangeably bound to tubulin. It appears possible then that the binding of glycerol by tubulin may substitute for, or otherwise preclude binding of, some of the accessory proteins found in our standard preparations.

Frigon and Timasheff (16) have shown that a double-ringed oligomer with an $s^{\circ}_{20,w}$ value of 42S forms from ion exchange-purified tubulin (>99% tubulin) in 0.01 M sodium phosphate at pH 7.0 and high $Mg^{++}$ concentrations. While it appears

safe to assume that this oligomer lacks tubulin-associated proteins and is different from our 30S species, we cannot exclude the possibility that it has the same arrangement of tubulin subunits as does the 36S species of the glycerol preparations.

Although our samples and those of Kirschner are similar in that several of the accessory proteins are the same, the relative concentrations of these proteins are different and their interactions with tubulin under depolymerizing conditions result in distinguishable oligomers. It is possible that the reported differences in microtubule self-assembly mechanism might derive from these inherent differences in the properties of the tubulin oligomers.

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REFERENCES:

1. Borisy, G.G. and Olmsted, J.B. (1972) Science 177, 1196-1197.
2. Olmsted, J.B., Marcum, J.M., Johnson, K.A., Allen, C.A. and Borisy, G.G. (1974) J. Supramolec. Struct. 2, 429-450.
3. Kirschner, M.W. and Williams, R.C. (1974) J. Supramolec. Struct. 2, 412-428.
4. Erickson, H.P. (1974) J. Supramolec. Struct. 2, 393-411.
5. Rebhun, L.I., Mellon, M.W., Jemiolo, D., Nath, J. and Ivy, N. (1974) J. Supramolec. Struct. 2, 466-485.
6. Kirschner, M.W., Williams, R.C., Weingarten, M. and Gerhart, J.C. (1974) U.S. Proc. Nat. Acad. Sci. 71, 1159-1163.
7. Weingarten, M.D., Suter, M.M., Littman, D.R. and Kirschner, M.W. (1974) Biochemistry 13, 5529-5537.
8. Borisy, G.G., Marcum, J.M., Olmsted, J.B., Murphy, D.B. and Johnson, K.A. (1975) Annals N.Y. Acad. Sci. 253, 107-132.
9. Erickson, H.P. (1975) Annals N.Y. Acad. Sci. 253, 60-77.
10. Dentler, W.L., Granett, S. and Rosenbaum, J.L. (1975) J. Cell Biol. 65, 237-241.
11. Shelanski, M.L., Gaskin, F. and Cantor, C.R. (1973) U.S. Proc. Nat. Acad. Sci. 70, 765-768.
12. Shapiro, A., Vinuela, E. and Maizel, J. (1967) Biochem. Biophys. Res. Comm. 28, 815-820.
13. Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606-2617.
14. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, B.J. (1951) J. Biol. Chem. 193, 265-273.
15. Detrich, H.W. III, Berkowitz, S.A., Kim, H. and Williams, R.C. Jr. Manuscript submitted to Biochem. Biophys. Res. Comm.
16. Frigon, R.P. and Timasheff, S.N. (1975) Biochemistry 14, 4567-4573.