TUBULIN DETERMINATION BY AN ISOTOPE DILUTION-VINBLASTINE PRECIPITATION METHOD

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INTRODUCTION

Estimation of tubulin content by colchicine binding has been hampered by the rapid decay of colchicine-binding activity in cell homogenates (Wilson, 1970). This problem has been confronted by Wilson (1970) and by Bamberg et al. (1973) by estimating colchicine binding at sequential times and extrapolating back to the time of homogenization.

In this paper, we report an alternative method for estimating tubulin concentration in tissue homogenates. It is an isotope dilution method: in vitro labeled tubulin is added to a homogenate, and dilution by endogenous tubulin is estimated from the specific activity of tubulin, which is recovered by vinblastine precipitation and further purified and quantitated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. While this method also may be complicated by tubulin denaturation, it is a useful complement to present procedures, since it does not depend on colchicine-binding activity. Moreover, the use of an internal standard corrects for differential loss or functional alteration of tubulin during preparative procedures.

Using this technique, we have compared tubulin content of unfertilized eggs, 4-cell embryos, and gastrulae of the clam, Spisula solidissima. Tubulin levels appear relatively constant throughout early development in this species. Our results are in accord with those of Raff et al. (1971) obtained from colchicine binding studies of Arbacia early embryos.

Total tubulin content in Spisula was found by our technique to be 2.0–2.4 \times 10^{-4} \mu g per egg or embryo, or approximately 3.3% of the total cell protein.

MATERIALS AND METHODS

A general abbreviated description of the entire method is presented first, with alphabetical reference to ensuing detailed descriptions.

Washed Spisula solidissima embryos were homogenized (A) upon addition of in vitro labeled sperm tail tubulin (B). The homogenate was centrifuged at 27,000 g for 15 min and the resulting supernate centrifuged at 100,000 g for 2.5 h. The pellets and samples of the homogenate and all supernates were retained for monitoring of tubulin loss (C). Vinblastine precipitates of the 100,000 g supernate were prepared according to a modification of the technique described by Olmsted et al. (1970). To the 100,000 g supernate was added 0.05 Vol of 2 \times 10^{-2} M vinblastine sulfate (Eli Lilly and Company, Indianapolis, Ind.); this mixture was allowed to stand for 1 h at 0°C, and the precipitate pelleted at 50,000 g for 20 min. Samples of vinblastine precipitates and labeled and unlabeled sperm tail tubulin standards, containing known amounts of protein as determined by the Lowry method (Lowry et al., 1951), were dissolved in electrophoresis sample buffer (D). Three different known amounts of unlabeled standard and duplicate samples of each vinblastine precipitate were run on 8% polyacrylamide gels containing 0.1% SDS (E). After staining the gels with Coomassie Brilliant Blue, the amount of tubulin was estimated by
densitometry of the respective tubulin bands and comparison to the standard gels. Tubulin radioactivity was determined by scintillation counting of 1-mm gel slices (F). After correction for counting efficiencies, a recovery factor was calculated for the labeled tubulin internal standard in each sample gel. The amount of tubulin in the original homogenate was estimated from the recovery factor and the amount of protein in the tubulin band.

A. Culture of Embryos and Preparation of Homogenates

Eggs from dissected ovaries of *Spioa solidissima* were filtered through cheese cloth and washed three times by settling through pasteurized sea water (PSW). 3 ml of packed eggs were suspended in 30 ml of PSW and divided into 10- and 20-ml aliquots. The 20-ml aliquot was diluted to 600 ml with PSW and the eggs fertilized by addition of 6.5 ml of a 0.5% sperm suspension. After 5 min the fertilized eggs were separated from sperm by low speed centrifugation, resuspended in 600 ml of PSW, and allowed to develop at 23°C with continuous stirring. Percent fertilization, estimated from germinal vesicle breakdown, was 98-99% with no evidence of polyspermy. Immediately before harvest, duplicate samples were collected from stirred suspensions with Eppendorf pipets and transferred to glass slides where all eggs present were counted. Sample sizes were adjusted to yield between 100 and 200 embryos per sample. 4-cell stage embryos and gastrulae were harvested at appropriate intervals from 300 ml of the culture by low-speed centrifugation. These embryos and the remaining unfertilized eggs were washed once in 9 ml and resuspended to 5 ml of SMT buffer (0.24 M sucrose, 10 mM Tris, pH 7.0, 20 mM MgCl2; from Olmsted et al., 1970). After addition of 100 µl of labeled tubulin, they were homogenized with 25 passes in a Dounce homogenizer; no intact cells were observable microscopically.

B. In Vitro Labeling of Tubulin Isolated from Arbacia punctulata Sperm

Sperm tail tubulin was prepared according to the method of Stephens (1970) with two modifications: (a) sperm tails were separated from heads and whole sperm by centrifugation after layering over sucrose, as described by Renaud et al. (1968); and (b) final solubilization of B-tubulin was accomplished by pipetting 2 ml of outer fiber suspension vigorously for 5 min at 37-40°C. Tubulin was labeled in vitro according to the method of Rice and Means (1971). The tubulin solution was adjusted to pH 9.0 with 0.1 N NaOH and to a final protein concentration of 1 mg/ml. The following additions were made to 1 ml at 4°C: 100 µl of 0.04 M [3H]formaldehyde (New England Nuclear, Boston, Mass., 77.6 mCi/mmol), followed by four 20-µl aliquots of sodium borohydride (5 mg/ml) at 30 s, and an additional 100 µl at 1 min. The solution was then run through a Sephadex G-25 (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) column at 4°C, eluted with 10 mM Tris, pH 8.0, to separate protein from unbound tracer. The 1-ml fraction of highest A280 was used as the marker tubulin preparation. Protein concentration in this sample was estimated by the Lowry method as 0.75 mg/ml (bovine serum albumin used as standard). By trichloroacetic acid (TCA) precipitation, it was determined that the protein specific activity was 4,670 cpm/µg. Freshly prepared marker tubulin was used immediately for the vinblastine precipitation experiments to avoid denaturation.

C. Monitoring of Tubulin Losses

1 ml of 10% TCA was added to 25-100 µl aliquots of homogenates, supernatants, and pellets suspended in 1% SDS. The precipitate was collected on Millipore filters (Millipore Corp., Bedford, Mass.) and washed with ethanol. The filters were dried and incubated for 2 h with 0.3 ml of protosol (New England Nuclear) in scintillation vials. To each vial was added 8 ml of a scintillation fluid containing 42 ml Liquifluor (New England Nuclear), 200 ml Scintisol (Isolab, Inc., Akron, Ohio), and 900 ml of toluene made 0.2% in acetic acid. Radioactivity was measured with a Beckman LS-250 scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

D. Preparation of Samples for Gel Electrophoresis

Samples containing 7.5-100 µg of protein were brought to 50 µl with 1% SDS, and 25 µl of the sample buffer was then added (0.15 g SDS, 5.4 g twice recrystallized urea, 3.0 ml glycerine, 0.6 mg bromphenol blue, 0.33 ml 2-mercaptoethanol, 11.58 g NaH2PO4·HOH, and 31.29 mg Na2HPO4·10H2O brought to 10 ml with distilled water). The mixtures were heated to 90-95°C for 5 min, shaken manually, and heated again.

E. SDS-Polyacrylamide Gel Electrophoresis

Gels were prepared as follows: 13.5 ml of Solution A (17.78 g of acrylamide and 0.48 g of N,N'-methylene-bis-acrylamide brought to 100 ml with distilled water) and 15 ml of Solution B (7.8 g of NaH2PO4·HOH, 22.55 g Na2HPO4, and 2.0 g twice recrystallized SDS in 1,000 ml of distilled water) were mixed and degassed briefly. Then 15 mg of ammonium persulfate in 1.5 ml of distilled water and 15 µl of N,N'-
N',N'-tetramethylethylene-diamine were added, and the mixture used to prepare gels. Running buffer was prepared from 900 ml of Solution B diluted 1:1 with distilled water. Gels were stained with 0.01% Coomassie Brilliant Blue in methanol:acetic acid:water (5:1:5) after 1 h fixation in 7.5% TCA, and destained with 7.5% acetic acid for 2 days.

F. Densitometric Determination of Tubulin Content and Estimation of Radioactivity in Gels

Gels containing known amounts of standard unlabeled and labeled protein were scanned with a Gilford spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). A standard curve for tubulin content of gels was produced by cutting out and weighing the peak of each gel scan from trough to trough. With the amount of protein used, the nonlinearity of the Coomassie Blue Stain was not bothersome (Fig. 2). Since both the amount and the specific activity of tubulin were comparable in the unknown samples (Table II), the relative estimated values of tubulin content per embryo are not in serious error; by comparing the percent total counts in the tubulin band vs. percent total absorbance in the tubulin band for the standard, we estimate that our reported values may underjudge the true values by no more than 30% because of the nonlinearity of the stain. Somewhat more accurate, although less sensitive, estimates could be obtained by the use of fast green (Gorovsky, et al., 1970). To correct for non-tubulin contamination of the standard, the amount of protein loaded on the gel was multiplied by the ratio of peak weight to total scan weight, giving the amount of protein in the tubulin band. For each vinblastine preparation, two quantitatively different samples were analyzed; the tubulin content of each was estimated from the standard curve and the radioactivity of the peak (trough to trough, cut in 1-mm slices) was determined after overnight incubation with 8 ml of a fluid composed of 42 ml Liquifluor, 970 ml toluene, and 30 ml protosol.

RESULTS

Distribution of Marker Tubulin during the Preparative Procedure

The distribution of exogenous marker tubulin was monitored during the fractionation of all samples. Table I shows the results, which were similar for all three developmental stages. A significant fraction of the marker radioactivity, approximately one-third was recovered in the low-speed pellet of the original homogenate. Since the marker does not pellet to a significant extent when processed under the same conditions but in the absence of tissue homogenate (with or without magnesium in the homogenizing buffer) we may attribute this loss to polymerization in the extract, passive adsorption to cell organelles, or denaturation induced by tissue constituents. A sedimentable tubulin fraction was also observed by others, using the colchicine-binding assay (Wilson, 1970; Weisenberg, 1972). An additional small fraction pelleted during the high-speed centrifugation. Because of this pelleting phenomenon, plus unavoidable losses, only approximately 40 ± 5% of the original radioactivity remained in the supernate to which vinblastine was added.

After vinblastine action, the radioactivity distributed in a ratio of approximately 2:1, supernate:pellet (range, 1.5 ± 2.5:1). Similar results were obtained with marker tubulin precipitated with vinblastine in the absence of tissue extract but in the presence of cold tubulin of concentration equivalent to that calculated for the homogenate. The results probably represent incomplete precipitation rather than further purification of the labeled monomer. Incomplete precipitation of tubulin under our conditions was to be expected. In particular, a temperature of 0°C (selected to minimize denaturation) is known to be suboptimal for vinblastine precipitation (Wilson et al., 1970). In any case, a rather constant proportion of the added marker was recovered in the vinblastine pellets of all stages (10-13%; Table I).

Electrophoretic Analysis of the Marker Protein

Much of the marker protein, both labeled and unlabeled, migrates as a single band upon SDS-polyacrylamide gel electrophoresis (Fig. 1). This major component results from the solubilization of the B-tubule of the outer doublet (Stephens, 1970), has the characteristic molecular weight of tubulin, and under appropriate buffer conditions, displays colchicine-binding activity (Wilson and Meza, 1972). As expected, in the labeled preparation the bulk component was coincident with the predominant radioactivity peak (Fig. 1).

As described in the Materials and Methods, data such as those in Fig. 1 were used to construct a standard curve for electrophoretically separated tubulin (Fig. 2). The abscissa of this curve assumes no losses of the sample loaded and is corrected for the non-tubulin constituents of the sample (Fig. 1).
**Table I**

*Distribution of Labeled Marker Tubulin during Fractionation of Spisula Homogenates*

| Homogenate | UF 100% (334,200 cpm) | 4C 100% (328,000 cpm) | G 100% (374,180 cpm) |
|------------|------------------------|------------------------|------------------------|

**Low-speed centrifugation**

| LS pellet | 31.9% | 33.7% | 37.8% |
|-----------|-------|-------|-------|

| LS supernate | 52.8% | 64.1% | 48.3% |

**High-speed centrifugation**

| HS pellet | 5.5% | 6.2% | 11.3% |
|-----------|------|------|-------|

| HS supernate | 40.1% | 45.5% | 35.8% |

| VB pellet | 13.2% | 10.6% | 10.0% |
|-----------|-------|-------|-------|

| VB supernate | 19.8% | 25.4% | 16.6% |

* For each of the three developmental stages, unfertilized eggs (UF), 4-cell embryos (4-C) and gastrulae (G), the counts recovered for each fraction are expressed as % of the total counts in the initial homogenate.

by multiplying the µg of protein loaded onto the gel by the ratio of tubulin peak absorbance to total gel absorbance.

**Electrophoretic Analysis of Vinblastine Precipitates**

Fig. 3 shows the electrophoretic profiles of vinblastine precipitates from three developmental stages. A single major component is apparent, coincident with the marker tubulin.

**Calculation of Tubulin Content per Embryo**

From data such as those shown in Fig. 3, and with the use of the standard curve (Fig. 2), the content of detectable tubulin per embryo was calculated (Table II). The content proved essentially constant at approximately $2 \times 10^{-4}$ µg per individual, from unfertilized egg to gastrula (Fig. 4).

The micrograms of tubulin in each homogenate ($8 \times 10^6$ eggs/embryos) was calculated as follows:

$$
\text{µg protein in gel tubulin peak} = \frac{\text{cpm in gel tubulin peak}}{\text{cpm in homogenate} \times F} - C
$$

Where $F$ is a correction factor for non-tubulin labeled components in the marker, losses during layering of samples and electrophoresis, and differences in counting efficiencies; it was calculated.
from the data of Fig. 1 upper two panels as

\[ F = \frac{\text{cpm in tubulin peak of labeled standard gel}}{\text{cpm in sample loaded on labeled standard gel}} \]

and found to be 0.41. C is a correction for the amount of tubulin contributed by the marker.

**Figure 2** Standard curve for tubulin, estimated from densitometric scans of marker gels (Fig. 1).

**Figure 3** Electropherograms of vinblastine precipitates from homogenized unfertilized eggs (UF), 4-cell embryos (4C) and gastrulae (G) of Spisula. The data represent the smaller of two samples analyzed. The marker tubulin is indicated by the radioactivity profile.
Time After Fertilization (Fours)

FIGURE 4

Tubulin content of Spisula at early stages of development. The bars indicate the standard error of the mean. See also Table II.

C = \frac{\text{cpm of labeled standard in homogenate}}{\text{specific activity of labeled standard}}

The specific activity of our preparation was 4,670 cpm/µg, and C was approximately 3% of the calculated tubulin content of the homogenate.

DISCUSSION

The Tubulin Content of Spisula

Our observation of a constant tubulin content in early Spisula embryos is consistent with and extends Weisenberg's (1972) observations that colchicine-binding activity in eggs of this species remains relatively constant during fertilization and the first meiotic division. Likewise, Raff et al. (1971) have found that there is a relatively constant level of colchicine-binding activity in sea urchin embryos from unfertilized egg through gastrula. We find that tubulin comprises approximately 3.3% of the 6.7 ng of total egg protein (determined by the Lowry method). Raff et al. (1971) did not attempt to quantitate tubulin per embryo, but did note that vinblastine precipitates 2–5% of the total embryo protein in Arbacia. Since our results indicate that a large fraction of the vinblastine precipitated from embryos is tubulin, and that vinblastine may not precipitate all the tubulin in a homogenate, the observation of Raff et al. (1971) is compatible with our results.

Although the marker tubulin was freshly prepared and used immediately after labeling, we cannot exclude the possibility that a fraction of it was resistant to vinblastine precipitation because of denaturation. This would lead to an overestimate of the absolute value of tubulin content in the samples. The process of labeling and recovering the marker did not in itself alter the precipitability of tubulin by vinblastine (unpublished observations) nor did it change its purity. Since all samples were processed simultaneously, relative estimates of their content, and thus the conclusions about tubulin constancy, would be unaffected.

The Tubulin Quantitation Method

The method described in this report is based on the assumption that labeled sperm tail tubulin behaves in a cell homogenate like the endogenous cytoplasmic tubulin. In view of the overall similarity in amino acid composition of different tubulins (Stephens, 1971), this is not entirely unreasonable as a starting assumption. It could be tested by appropriate mixing experiments, in-

TABLE II

Tubulin Content of Spisula Embryos

| A | µg protein in tubulin band | B | cpm in tubulin band | C | Specific activity (cpm/µg) tubulin | D | cpm in original homogenate | F | µg tubulin in original homogenate | G | µg tubulin/embryo |
|---|-----------------|---|-----------------|---|------------------|---|------------------|---|------------------|---|-----------------|
| Unfertilized egg | 5.0 | 403 | 80.6 | 334,200 | 1,700 | 1,574 ± 53 | 2.0 × 10^{-4} |
| 10.0 | 860 | 80.6 | 334,200 | 1,593 | ± 0.16 × 10^{-4} |
| 4-Cell stage | 6.8 | 425 | 62.5 | 326,000 | 2,152 | 1,955 ± 125 | 2.4 × 10^{-4} |
| 12.8 | 906 | 70.8 | 326,000 | 1,900 | ± 0.16 × 10^{-4} |
| Gastrulae | 6.4 | 532 | 83.1 | 374,180 | 1,845 | 1,766 ± 1 | 2.2 × 10^{-4} ± 0 |
| 12.8 | 1,063 | 83.0 | 374,180 | 1,847 | |

* Data as in Fig. 3, calibrated with the standard curve (Fig. 2).
† Column D divided by column C and multiplied by the recovery factor F (see text).
‡ Average of the two values in column E minus the amount of exogenous marker.
§ Column F divided by the number of embryos per homogenate.

Figure 4 Tubulin content of Spisula at early stages of development. The bars indicate the standard error of the mean. See also Table II.

A µg protein in tubulin band
B cpm in tubulin band
C Specific activity (cpm/µg) tubulin
D cpm in original homogenate
F µg tubulin in original homogenate
G µg tubulin/embryo

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volve quantitatively variable mixtures of labeled purified tubulins from various sources, unlabeled samples of the same, and various crude preparations.

Before such a rigorous test is attempted, it would seem appropriate to eliminate the major difference between marker and endogenous tubulins. At present, it would be possible to hypothesize that the two differ in the extent to which they polymerize and thus are differentially sedimented before vinblastine treatment. We suspect that this is not the case, because of the striking constancy of tubulin content even in the ciliated gastrulae; ciliary tubulin might be expected to bear greater resemblance to the marker than to the remaining endogenous tubulin (Stephens, 1971). In any case, this objection might be met by using colchicine during homogenization and subsequent procedures, not for assay but to enhance tubulin depolymerization. (It is of interest to note here that the presence of vinblastine dramatically retards decay of colchicine-binding activity and there is no evidence of competition for binding sites (Wilson, 1970). Use of our method in experiments with and without colchicine in the homogenizing medium might also yield some interesting suggestions about the aggregation state of egg tubulin at different stages of development and complement similar studies using colchicine binding reported by Weisenberg (1972).

Finally, the method will become considerably more useful when methods are developed for stable storage of tubulin, with predictably low incidence of denaturation. At that point, labeled tubulin will become a reagent, and comparisons of different tissue extracts may no longer require simultaneous experiments.

Clearly, the method is still at its infancy. We offer it at this time as a prototype of a whole class of possible methods. For example, isotope dilution could also be combined with immunological recognition of tubulin perhaps in a method using matrix-bound antitubulin. Whether combined with antibody or vinblastine precipitation, isotope dilution offers the essential opportunity of reciprocal checks with the colchicine-binding assay. Despite its merits, colchicine binding shares the uncertainties of all functional (as distinct from chemical) determinations. Finally, the isotope dilution method could be adapted for estimation, in embryonic or other tissues, of any soluble protein which can be partially purified to the point that it can be recognized as a distinct peak in SDS gel electrophoresis.

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