Cytoplasmic Microtubule Proteins of the Embryo of *Drosophila melanogaster*†

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ABSTRACT: We have been able to purify, in bulk, the cytoplasmic microtubule proteins of eggs and embryos of *Drosophila melanogaster* by means of in vitro self-assembly of microtubules from subunits present in a high-speed supernatant fraction of eggs or embryos. This provides the first successful application of this method to purification of microtubule protein from a source other than vertebrate brain, and the first purification of insect microtubule proteins. Our electron micrographs show that the in vitro assembled microtubules are morphologically typical and apparently are comprised of the expected 13 protofilaments. The protein we obtain from such preparations binds [^{3}H]colchicine and has a sedimentation value of 6.4 S–6.9 S which is close to the predicted value for microtubule protein dimer. Both α- and β-microtubule proteins are evident in sodium dodecyl sulfate polyacrylamide electropherograms of the isolated proteins. The apparent molecular weights of these species on dodecyl sulfate polyacrylamide gels are 54,000 and 52,000, respectively. These values as well as the amino acid composition and N-terminal methionine of the *Drosophila* proteins are very closely comparable to microtubule proteins from other, unrelated organisms.

Cytoplasmic microtubules play a key role in eucaryotic cells in the establishment of cellular asymmetries and extensions, and in the accomplishment of chromosome separation in mitosis. However, unlike ciliary microtubules, cytoplasmic microtubules are extremely labile and have been difficult to isolate. The study of cytoplasmic microtubules has been greatly facilitated by the recent development of methods for the in vitro assembly of brain microtubule proteins (Borisy and Olmsted, 1972; Weisenberg, 1972; Shelanski et al., 1973).

In this paper we report the first successful purification of cytoplasmic microtubules from a system other than brain (embryos of the fruit fly, *Drosophila melanogaster*) by in vitro assembly and have determined the behavior of the soluble pool in early development to provide a basis for a genetic examination of the regulation of microtubule protein function.

Drosophila is an excellent organism for the study of the role of cytoplasmic microtubules in early embryonic development. The cell cycle in early *Drosophila* embryos is only 10 min long (Sonnenblick, 1950). With each cycle mitotic apparatuses must be dismantled and reassembled to provide for nuclear division. In the first 2 hr of development at 25° the embryo completes nine nuclear division cycles to become a plasmodium containing about 500 nuclei. Most of these nuclei migrate to the periphery of the embryo and complete three more nuclear divisions. This is followed by formation of the blastoderm stage and immediately after that by gastrulation and organogenesis. There is no investment of microtubule protein into cilia. However, during blastoderm formation cytoplasmic microtubules determine the shape and orientation of the elongating nuclei at the periphery of the embryo (Fullilove and Jacobson, 1971).

Further, *Drosophila* embryos can be obtained in large quantities and thus provide a source for the large scale preparation of insect microtubule protein.

Materials and Methods

Culture Methods. Drosophila melanogaster were raised in population cages. Agar and molasses trays covered with yeast were used to feed the flies and to collect eggs. Eggs from the trays were washed through a U.S. Standard Sieve Series (W.S. Tyler Co.) of 841, 420, and 125 μm. Eggs were collected with 0.01% Triton-X from the 125-μm sieve, and dechlorionated by gentle stirring in 2.75% sodium hypochlorite for 5 min. The eggs were then washed several times until contaminating feces and debris were removed (Alfageme et al., 1974).

Purification of Microtubule Protein. Embryos were homogenized at 4° in an equal volume of a buffer consisting of 0.1 *M* 2-(*n*-morpholino)ethanesulfonic acid (Mes), 1 m*M* ethylenediaminetetraacetate (EDTA) or ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetate (EGTA), 1 m*M* GTP, and 0.5 m*M* $MgCl_2$ at pH 6.4 as described by Weisenberg (1972). These homogenates were then centrifuged at 27,000*g* for 10 min to remove nuclei and unbroken cells, and then at 150,000*g* for 90 min to remove all mitochondria and ribosomes. The 150,000*g* supernatant was diluted with an equal volume of homogenization buffer containing 8 *M* glycerol, and incubated for 30 min at 25°. Following this, incubation mixtures were centrifuged at 100,000*g* for 60 min. Microtubule pellets from this first assembly step were resuspended in one-half the original volume of homogenization buffer at 4° and incubated for 30 min; then centrifuged at 150,000*g* for 90 min at 4°. The supernatant obtained could be used for further assemblies or other purification steps.

Analytical Procedures. Sodium dodecyl sulfate-urea acrylamide gels were prepared and run as described by Raff and Kaumeyer (1973). Each tube consisted of a 7.5% acrylamide running gel, and a 2.5% acrylamide stacking gel. Electrophoresis was performed at 1.25 mA/gel until the tracking dye had reached the end of the gel. Gels were stained overnight with 0.05% Coomassie Blue in 9% acetic

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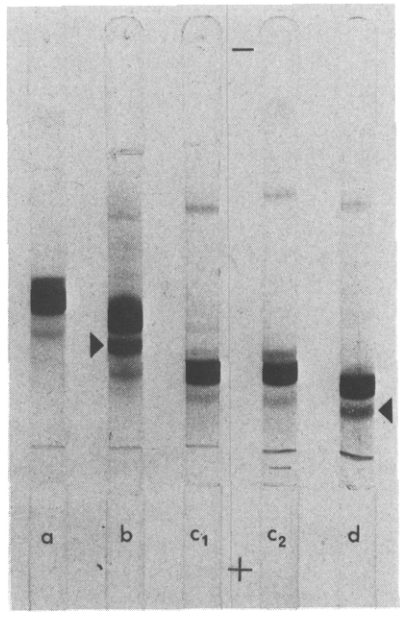

FIGURE 5: Gel electrophoresis patterns for heavy loadings of Lb a, Lb b, Lb c_1, Lb c_2, and pooled Lb d (from top to bottom) at pH 8.9.

mated at 7% of the total by scanning the gel spectrophotometrically. Figure 5 shows the gel patterns obtained at pH 8.9. The minor bands running toward the anode ahead of the main bands in Lb b and Lb d were heme-containing but the other trace contaminant bands visible in Figure 5 showed up only after staining. Lb c_1 and c_2 were electrophoretically indistinguishable either at pH 5.2, 7.0 (sodium dodecyl sulfate), or 8.9.

Amino Acid Analysis and Peptide Mapping of Lb c_1 and Lb c_2. The amino acid analyses for Lb c_1 and c_2 are shown in Table II, together with the literature values (Ellfolk, 1961a) for unfractionated Lb c. Lb c_2 appears to have one isoleucine residue additional to those in Lb c_1 but at least one aspartic acid, one glutamic acid, four alanine, three valine, and one leucine residue less than Lb c_1. Both proteins contain two tryptophan residues and this was confirmed by analyses on the intact protein which gave values of 2.0 for both Lb c_1 and Lb c_2. The mean values obtained for Lb c_1 and Lb c_2 agree with the literature value for unfractionated Lb c (Ellfolk, 1961a) for all but the serine, glycine, valine, and tryptophan data.

In addition to the origin spot there are about 14–18 peptide spots reactive to ninhydrin, though some spots are very weak in intensity (Figure 6). Resolution of all the possible peptides is incomplete under these conditions but is adequate for demonstrating differences between proteins. The peptide maps show some definite amino acid differences between Lb c_1 and Lb c_2. Peptides 14 and 15 in Lb c_2 do not occur in Lb c_1. Peptides 16 and 17 are weak and present only in Lb c_2. pH 3.5 electrophoresis of the neutral peptides indicated one further peptide difference between Lb c_1 and Lb c_2.

Circular Dichroism of Lb c_1 and Lb c_2. The CD spectra of the acetate complexes of the two Lb c components are recorded in Figure 7. Figure 7a shows the far-ultraviolet CD spectra and indicates that the secondary structure of Lb c_1 and c_2 is essentially the same; about 52% helix and 48% unordered structure. CD spectra for Lb c_1 and c_2 in the near-ultraviolet, which reflect the environments around the aromatic amino acids of proteins, are shown in Figure 7b

Table II: Amino Acid Analyses[a] of Lb c_1 and Lb c_2.

Amino Acid	Lb c_1 Mean	Lb c_1 Nearest Integer	Lb c_2 Mean	Lb c_2 Nearest Integer	Lb c[b]
Lys	13.05 (0.52)	13	12.79 (0.23)	13	13
His	2.19 (0.11)	2	1.97 (0.17)	2	2
Arg	1.00 (0.03)	1	0.96 (0.04)	1	1
Asp	12.55 (0.19)	12	11.04 (0.18)	11	12
Thr	6.92*	7	7.03*	7	7
Ser	9.80*	10	10.10*	10	11
Glu	15.14 (0.27)	15	13.75 (0.20)	14	14
Pro	4.61 (0.34)	5	4.65 (0.32)	5	5
Gly	7.00 (0.04)	7	7.26 (0.25)	7	8
Ala	22.88 (0.07)	23	19.19 (0.60)	19	21
Val	12.8*	13	10.21 (0.18)	10	13
Ile	4.84 (0.18)	5	5.85 (0.05)	6	6
Leu	12.94 (0.24)	13	12.16 (0.27)	12	12
Tyr	2.90*	3	2.90*	3	3
Phe	7.80 (0.39)	8	8.18 (0.09)	8	8
Trp	2.01	2	1.85	2	3

[a] The apoproteins of Lb c_1 and Lb c_2 (0.5 mg) were hydrolyzed (100 μl of 6 *M* HCl and 1% phenol, 110°, 24, 48, and 72 hr in vacuo) and analyzed with a Beckman 120 B analyzer. The values marked with an asterisk have been corrected for destruction of Ser, Thr, and Tyr and incomplete release of Val. Standard deviations are shown in parentheses. [b] Literature values for unfractionated Lb c (Ellfolk, 1961a).

and are indistinguishable from each other. Differences in the Soret region (Figure 7c) are barely significant. The possibility that one component is a denatured form or conformational variant of the other may therefore be discounted.

Discussion

Prior oxidation of the leghemoglobin extract with ferricyanide converted all heme protein components to their ferric forms. Subsequent ion-exchange chromatography then showed no evidence of the $Fe(II)O_2$ components which otherwise complicate the elution profile. Elimination of nicotinate (called X by Appleby, 1969b; Appleby et al., 1973a,b) from crude Lb by gel filtration at pH 9.2 also eliminated the double bands due to partial formation of Lb nicotinate hemochrome. Previously (Appleby, 1969b) these bands had complicated the ion-exchange elution profile. The use of a continuous, shallow acetate gradient with DE-52-cellulose then achieved a good resolution, not only of the previously reported components (Lb's a, b, c, d) but also demonstrated that Lb c and Lb d each have two components. Unlike the Lb d components, those of Lb c (c_1 and c_2) were quite well separated by the initial ion-exchange chromatogram. Further purification was achieved by a second run under similar conditions with a shallow acetate gradient. It was found important to maintain a constant low pH of 5.30 in both separations; for example, no resolution of the two Lb c components was observed in phosphate buffers at either pH 6.5 or 7.8. It is interesting to note that the resolution attainable in this ion-exchange chromatographic procedure is superior to that achieved in polyacrylamide gel electrophoresis even in the presence of acetate. In Figure 5 it is seen that the components Lb c_1 and Lb c_2 cannot be distinguished by gel electrophoresis at pH 8.9, or at pH 5.2 or 7.0 (not shown).

It has been conclusively shown by Cann and Goad (1964) and Cann (1969) that electrophoresis of proteins in carboxylic acid buffers can produce illusory additional components due to complexing between the protein and undissociated

Table I: Colchicine Binding during the Isolation of Microtubule Protein by Self-Assembly from Embryos of *Drosophila melanogaster.*

Step	Volume (ml)	Activity/ ml (cpm)	Total Activity (cpm)	Total Protein (mg)	Specific Activity (cpm/mg)	Yield (%)	Purification
Differential spin supernatant of a 27,000 *g* centrifugation of the homogenate	40	4.82×10^5	19.28×10^6	1036.8	18.5×10^3	100	1
First 150,000 *g* supernatant	28	5.06×10^5	14.17×10^6	411.8	34.4×10^3	74	2
First microtubule pellet (solubilized)	13	4.51×10^5	5.87×10^6	24.40	240.0×10^3	30.6	13
150,000 *g* supernatant from the centrifugation of solubilized microtubules	12	3.15×10^5	3.78×10^6	12.65	299.0×10^3	19	16

Table II: Binding of [^{3}H]Colchicine by *Drosophila* Embryos as a Function of Development.

Expt No.	Age of Embryos[a] (hr)	Binding[b] (cpm/mg of Protein)	Relative Binding
1	0.5	28,300	1.00
	1.5	27,000	0.95
	4.0	31,300	1.10
	5.0	31,300	1.10
2	1.0	27,200	1.00
	4.0	25,100	0.92
3	1.0	23,800	1.00
	3.0	24,000	1.01
4	1.0	42,900	1.00
	4.0	49,800	1.16

[a] Embryos were maintained at 25°. Ages represent the time since laying for each batch of embryos. Fertilization occurs shortly prior to laying. Cleavage is rapid in the period from laying to about 3 hr by which time the embryo completes blastoderm formation: gastrulation is complete at 4 hr and organogenesis has begun by 5 hr of development. Ages of embryos in experiment 1 are ±30 min. Ages of embryos in other experiments are ±60 min. [b] Values determined as described in Figure 3 using 60-min incubation of protein with [^{3}H]colchicine. [^{3}H]Colchicine used in experiment 4 had a higher specific activity than that used in the other experiments.

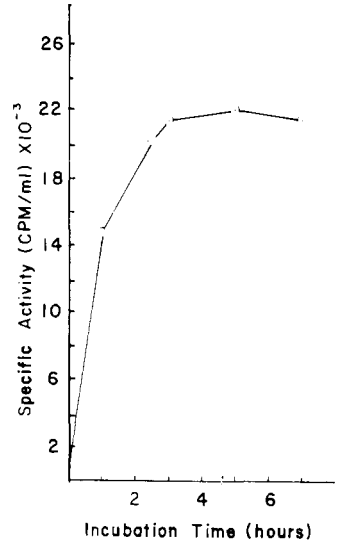

FIGURE 3: [^{3}H]Colchicine binding as a function of time of incubation. Embryos were homogenized in PMg buffer and centrifuged at 150,000*g*/90 min; 1.0-ml samples were incubated with 2.5×10^{-6} *M* [^{3}H]colchicine (2 Ci/g) for the indicated times.

Assembled microtubules or proteins precipitated with ammonium sulfate could be stored for over a month in 50% glycerol at −20° without loss of antigenic activity or change in electrophoretic pattern.

Colchicine Binding and Microtubule Protein Pool. We employed the binding of ^{3}H-labeled colchicine to follow purification and to determine possible changes in the size of the microtubule protein pool during early development. We have assumed that colchicine-binding activity in high-speed supernatant fractions represents only microtubule protein which has been shown to have this capacity in other systems (e.g., Weisenberg et al., 1968). Further, purified *Drosophila* microtubule protein (Figure 2 and Table I) binds [^{3}H]colchicine. There is, as yet, no documented case of [^{3}H]colchicine binding to a soluble, nonmicrotubule protein species. Figure 3 shows the binding of [^{3}H]colchicine as a function of time at 37°. Binding of [^{3}H]colchicine neared completion after 3 hr. Binding of [^{3}H]colchicine was dependent on protein concentration and was linear in the range of protein concentration used (0.27–1.3 mg/ml). These results are similar to those reported for microtubule proteins from sea urchin eggs (Borisy and Taylor, 1967), brain (Wilson, 1970), and from grasshopper (Wilson and Friedkin, 1967). We have measured the colchicine binding capacity at several stages of early development (0–5 hr) and found a constant level of binding, consistent with a constant pool of microtubule proteins (Table II).

Bamburg et al. (1973) reported that the half-life of colchicine binding activity of chick brain microtubule protein changed significantly during development, and that this could produce artifacts in the determination of colchicine binding levels measured at different points in development. To eliminate this phenomenon as a possible source of error in determination of pool size during *Drosophila* development, we measured the decay of colchicine binding capacity at 37° in 150,000*g* supernatant fractions of 1 hr (cleavage) and 4.5 hr (post-gastrulation) old embryos (Figure 4). There was no measurable difference in decay rate. Preparations from both stages exhibited a half-life of decay of 6.6 hr.

The pool size determinations shown in Table II represent data from 1-hr incubations of high-speed supernatant fractions with [^{3}H]colchicine. Incubations of this length were used because colchicine-binding was approximately linear over this interval, and corrections for decay of colchicine-binding capacity were unnecessary. Our minimum estimate for the size of the microtubule protein pool in *Drosophila* eggs is 10×10^{-9} g/embryo, which represents 0.4% of the total protein of the embryo or about 2.7% of the proteins remaining soluble after centrifugation at 150,000*g*. This is based on recovery of purified microtubule protein measured

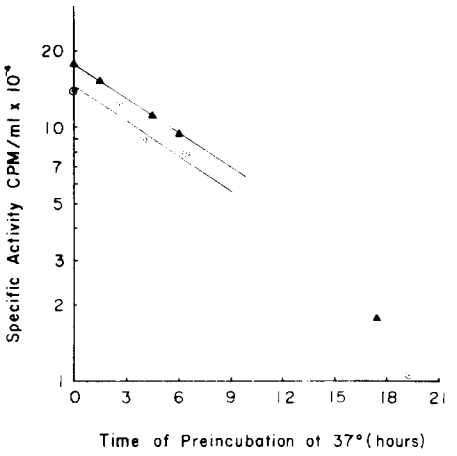

FIGURE 4: Decay of [^{3}H]colchicine binding activity at 37° of supernatants prepared from 1.0-hr and 4.5-hr old embryos. Embryos 1.0 (○) or 4.5 hr (△) old were homogenized, and 150,000g/90-min supernatants of the respective homogenates were incubated at 37°. At the indicated times 1.0-ml samples were incubated in 2.5 × 10^{-6} M [^{3}H]colchicine (2 Ci/g) for 60 min.

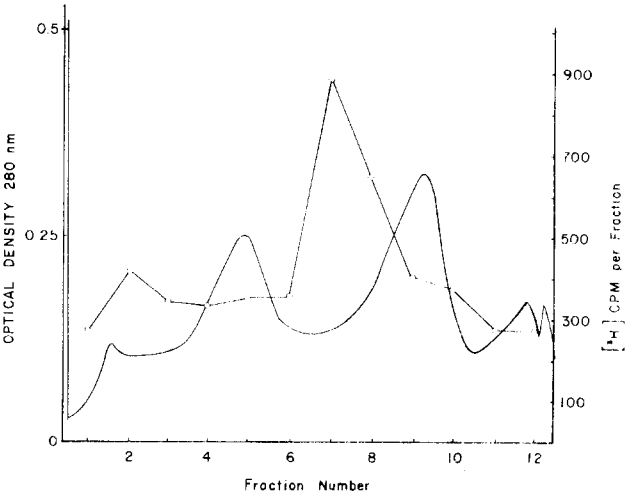

FIGURE 5: Sedimentation coefficient of the native microtubule protein. In vitro purified microtubule protein, catalase, and bovine serum albumin were centrifuged on a 6–20% sucrose gradient at 200,000g for 16.5 hr. Absorbance at 280 nm was measured by pumping the gradient through a Gilford flow cell at 1 ml/min; 1.0-ml fractions of the gradient were incubated in 2.5 × 10^{-6} M [^{3}H]colchicine (2 Ci/g) for 60 min. The bottom of the gradient is at the left. Absorbance (– –); ^{3}H cpm (○).

by the Lowry method (1951), and assumes no losses in purification. Losses were estimated by [^{3}H]colchicine binding (Table I) at various stages of purification. An estimate of the microtubule protein pool incorporating corrections for losses yields a figure of 41 × 10^{-9} g/embryo which equals 1.6% of total protein and 11% of proteins remaining in the 150,000g supernatant.

Molecular Weights. The apparent molecular weights of the monomeric subunits from in vitro assembled microtubules were determined by electrophoresis on polyacrylamide gels containing dodecyl sulfate and urea (Figure 2). This gel system resolves *Drosophila* microtubule proteins into two bands. The α (lower mobility) band had an apparent molecular weight of 54,000 ± 2000 and the β band 52,000 ± 2000. These values are identical with those previously reported for sea urchin microtubule proteins with this system (Raff and Kaumeyer, 1973; Raff et al., 1975). The ratio of α to β subunits recovered from in vitro assembled microtubules was somewhat variable, but the average ratio from several preparations was α/β = 1.2:1.0. Since we have not yet been able to isotopically label *Drosophila* embryo proteins in vivo, this ratio is based on Coomassie Blue staining. However, equimolar staining of α and β subunits cannot be assumed. Bryan (1972) reported that the color yield of the α band of sea urchin microtubule protein with Coomassie Brilliant Blue was 10–15% greater than that of the β band. Bibring and Baxandall (1974) have reported that the relative staining of the α and β bands is concentration dependent.

The native subunit which binds colchicine and is capable of assembly into microtubules has been found in other systems to be a dimer of about 120,000 daltons with a sedimentation value of 6 S (Olmsted and Borisy, 1973). We have determined the s value of native *Drosophila* microtubule proteins obtained from in vitro assembled microtubules. Density gradient cocentrifugation of *Drosophila* protein with catalase (11.4 S) and bovine serum albumin (4 S) yielded a sedimentation value of 6.4 S–6.9 S, which corresponds to an apparent molecular weight of 128,000 (Figure 5). Published values range from 110,000 to 130,000 (Stephens, 1971).

Amino Acid Composition. The amino acid composition of reduced, carboxymethylated protein (Crestfield et al., 1963) is shown in Table III along with previously published values for cytoplasmic microtubule proteins from other systems. Triplicate analysis of the same protein and analysis from two different preparations agreed. The serine and threonine contents were corrected for hydrolytic loss as described in Methods. The composition of *Drosophila* microtubule protein does not differ in any significant respect from the composition of other microtubule proteins. The N-terminal amino acid was found to be methionine. This is in agreement with reports of methionine as the N-terminal amino acid of sea urchin sperm tail and brain microtubule proteins (Luduena and Woodward, 1973), and with the finding (J. Brandis, unpublished data) of methionine, by the dansyl chloride method, on the N-terminus of sperm tail

Table III: Amino Acid Compositions of Various Microtubule Proteins (mol %).

Amino Acids	*Drosophila*	Source					
		a	*b*	*c*	*d*	*e*	*f*
Lys	5.8	3.9	6.2	4.6	4.3	3.9	5.4
His	2.8	2.4	2.3	2.7	2.4	2.2	2.5
Arg	5.0	4.4	5.3	4.9	5.1	4.7	5.7
Asp	10.9	10.3	9.8	10.6	11.0	11.1	10.9
Thr	5.8	6.3	6.1	5.4	5.4	6.8	6.0
Ser	5.8	5.9	5.2	6.8	4.4	7.3	5.0
Glu	12.9	14.2	13.2	13.0	13.0	13.5	13.7
Pro	5.2	5.2	5.0	5.3	4.5	4.7	5.1
Gly	8.1	7.9	7.9	8.4	8.4	9.1	7.9
Ala	8.0	7.5	7.8	7.3	7.5	7.6	7.9
Val	7.0	6.3	6.5	6.7	7.3	7.9	6.7
Met	2.8	2.3	2.2	2.8	3.2	1.6	2.3
Ile	4.8	4.5	4.9	4.5	4.9	4.4	5.0
Leu	7.9	7.7	8.5	7.3	7.5	7.2	7.9
Tyr	3.2	3.9	2.9	3.4	3.7	3.6	3.6
Phe	4.2	4.8	3.9	4.1	4.0	4.8	4.5

[a] Pig brain (Weisenberg et al., 1968). [b] Mouse neuroblastoma (Olmsted et al., 1970). [c] Squid axone (Davison and Huneeus, 1970). [d] Bovine brain (Arai and Okuyama, 1973). [e] Chick embryo brain (Bryan and Wilson, 1971). [f] Sea urchin sperm tail (Stephens, 1970).

microtubule proteins from three species of sea urchin (*Arbacia punctulata, Strongylocentrotus purpuratus,* and *Lytechinus pictus*).

Discussion

We have employed the in vitro assembly technique to purify microtubule proteins from eggs and embryos of *Drosophila melanogaster.* The cytoplasmic microtubule proteins of *Drosophila* eggs are very similar to well-characterized microtubule proteins from other unrelated organisms. The amino acid composition is almost identical with that of the cytoplasmic microtubule proteins of mammalian and chick embryo brain and to that of the flagellar microtubule proteins of sea urchin sperm. Further similarities reside in the [^{3}H]colchicine binding properties and in the molecular weights of the dimer and subunits. Like all other microtubule proteins analyzed to date, *Drosophila* proteins have an N-terminal methionine. Finally, the microtubules produced by in vitro assembly have, seen in cross-sections, the expected 13 subunit structure. We have no sequence data for *Drosophila* microtubule proteins. Luduena and Woodward (1973) have published preliminary sequence studies of brain and sea urchin microtubule proteins which indicate strong sequence conservation which is consistent with the morphological and functional conservation of microtubules.

It has been suggested that the in vitro assembly of vertebrate brain microtubule may involve nucleation centers (Olmsted and Borisy, 1972) or high molecular weight accessary proteins (Sloboda et al., 1975). We have indirect evidence that such nucleation sites may play a role in the in vitro assembly of *Drosophila* microtubules. While we have been successful in assembling microtubules from the first 150,000*g* supernatants of homogenized eggs, we have found that when these microtubules are resolubilized to yield a sample at least 19 times more enriched for [^{3}H]colchicine binding activity than the 150,000*g* supernatant, more than 90% of this resolubilized protein is not capable of assembling again. The fact that this protein binds [^{3}H]colchicine with a very high specific activity suggests that nucleation sites that were available for the first assembly either were not resolubilized, or were destroyed. The small number of microtubules capable of reassembling a second time show a high proportion of "C" and "S" shaped cross-sections, which indicates only partial assembly. We have not observed possible high molecular weight accessory proteins in our electrophoretic gels.

Embryos utilize a microtubule protein pool for the assembly of microtubule containing organelles during development (Raff et al., 1975). Our data on the [^{3}H]colchicine binding capacity of various stages of development indicate that this pool remains constant throughout early development in *Drosophila.* This constancy has also been observed in embryos of the very dissimilar organisms *Spisula* (a clam) (Burnside et al., 1973) and *Arbacia* (a sea urchin) (Raff and Kaumeyer, 1973).

The data presented in this paper were obtained from wild type *Drosophila.* An examination of mutants defective in microtubule protein function will provide a basis for analysis of the regulation of the microtubule protein pool and the assembly of microtubules in development.

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