Developmental and comparative aspects of brine shrimp tubulin

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Tubulin from embryos of the brine shrimp *Artemia* has been purified to apparent homogeneity by chromatography on phosphocellulose P11 and DEAE-cellulose, $(\text{NH}_4)_2\text{SO}_4$ fractionation and assembly–disassembly of microtubules. Peptide mapping indicated that *Artemia* and bovine brain tubulin were very similar in spite of differences in the electrophoretic behaviour of tubulin from these two organisms. Isoelectric focusing and two-dimensional gel electrophoresis were used to resolve and identify several *Artemia* isotubulins. The isotubulin composition and the quantity of tubulin did not change during pre-emergence development of *Artemia* embryos. Formation of microtubules with tubulin purified from embryos at different stages of development did not require glycerol or microtubule-associated proteins and formation of structurally normal microtubules was actually hindered by glycerol and Mg^{2+} . The characteristics of *Artemia* tubulin, in concert with the unusual life history of *Artemia*, suggest that this organism will be very useful for the study of tubulin gene expression and tubulin utilization during embryo development.

Microtubules are cylindrical organelles involved in chromosome segregation, motility, axonal transport, secretion and programmed modifications of cell shape (Roberts & Hyams, 1979; Cappuccinelli & Morris, 1982). The major structural protein of microtubules is tubulin, a heterodimer composed of α - and β -tubulin each with a molecular mass of about 50000 Da. Tubulin polymerization may depend on microtubule-associated proteins (MAPs), a group of proteins which copurify with tubulin during assembly–disassembly of microtubules (see Vallee & Davis, 1983, for review). There are, however, several examples of MAPs-independent microtubule assembly, especially for tubulin from non-neural sources (Kuriyama, 1977; Farrell & Wilson, 1978; Doenges *et al.*, 1979; Keller & Rebhun, 1982; Detrich & Wilson, 1983; Suprenant & Rebhun, 1983).

The use of peptide mapping, which has revealed the phylogenetic inter-relationships of tubulin from various organisms (Little *et al.*, 1981; Ludueña & Little, 1981), amino acid sequence analysis (Ponstingl *et al.*, 1982) and recombinant DNA techniques (Alexandraki & Ruderman, 1981; Ginzburg *et al.*, 1981; Valenzuela *et al.*, 1981; Cleveland & Kirschner, 1982; Wilde *et al.*,

1982a,b,c; Mischke & Pardue, 1982; Kalfayan & Wensink, 1982; Brunke *et al.*, 1982b; Hall *et al.*, 1983; Lopata *et al.*, 1983; Neff *et al.*, 1983) has shown that α - and β -tubulin are conserved polypeptides which are products of multiple, non-identical tubulin genes. Tubulin microheterogeneity is manifested by the appearance of tissue-specific tubulins in *Drosophila* testis (Kemphues *et al.*, 1980, 1982) and rat liver (Denoulet *et al.*, 1982b), while organelle-specific tubulins occur in *Chlamydomonas* (Brunke *et al.*, 1982a,b; McKeithan *et al.*, 1983), *Polytomella* (McKeithan & Rosenbaum, 1981; McKeithan *et al.*, 1983), *Naegleria* (Lai *et al.*, 1979), scallops (Stephens, 1981) and sea urchins (Bibring *et al.*, 1976). Developmental modification of tubulin isoforms has been studied in liver (Denoulet *et al.*, 1982b), brain (Dahl & Weibel, 1979; Denoulet *et al.*, 1982a), axolotl (Raff & Raff, 1978), *Drosophila* (Mischke & Pardue, 1982; Kalfayan & Wensink, 1982; Raff *et al.*, 1982), *Naegleria* (Lai *et al.*, 1979), *Chlamydomonas* (Brunke *et al.*, 1982a,b; Silflow *et al.*, 1982; McKeithan *et al.*, 1983) and a variety of cells in culture (Edde *et al.*, 1981; Cleveland *et al.*, 1981; Cleveland & Kirschner, 1982; Spiegelman & Farmer, 1982).

If, as suggested by the study of tubulin microheterogeneity, microtubule assembly and function are influenced by the tubulin isoforms in the cell,

Abbreviations used: MAPs, microtubule-associated proteins; SDS, sodium dodecyl sulphate; Pipes, 1,4-piperazinediethanesulphonic acid.

then precise regulation of tubulin multi-gene families is critical to cell development and function. To examine this idea, to ascertain the role of MAPs in microtubule assembly and embryo development, and to extend our knowledge of tubulin phylogenetic inter-relationships we have chosen to study the brine shrimp, *Artemia*. Brine shrimp exhibit an unusual life history (Hentschel & Tata, 1976; Persoone *et al.*, 1980) conducive to the study of tubulin gene expression during embryogenesis. Encysted *Artemia* gastrula undergo a relatively synchronous development characterized by the synthesis of polypeptides on newly formed polyosomes (Golub & Clegg, 1969) and, most importantly, the absence of DNA synthesis and cell division (Olson & Clegg, 1978). The period of embryonic development without cell division, termed pre-emergence development as it occurs within the intact cyst, permits study of developmental processes apart from changes associated with mitosis, a microtubule-dependent event. Thus, potentially complicating effects of simultaneous changes in tubulin and MAPs populations for mitosis versus cell differentiation are reduced.

As described in this paper, the first step in our study was to purify *Artemia* tubulin to apparent homogeneity. *Artemia* and bovine tubulin differ in isoelectric points, migration on SDS/polyacrylamide gels and assembly characteristics, but their peptide maps are unexpectedly very similar. The isotubulin population in *Artemia* embryos does not change during the first 15h of pre-emergence development, nor does there appear to be a major increase in the amount of tubulin in the embryos. The results of our experiments raise a number of interesting points regarding the synthesis, utilization and evolutionary history of *Artemia* tubulin.

Experimental

Materials

GTP as sodium salt and Pipes as free acid were purchased from Sigma. All other chemicals were reagent grade. Phosphocellulose (P11) and DEAE-cellulose (DE52) were from Whatman. *Artemia* embryos were supplied by San Francisco Bay Brand, Newark, CA, U.S.A. Taxol was kindly given by Dr. Matthew Suffness, Natural Products Branch, NCI, Bethesda, MD, U.S.A.

Embryo development

Dormant *Artemia* embryos (40g dry wt.) were hydrated in 200ml of cold water for at least 3h, followed by washing with cold water on a Buchner funnel. For development, 125g wet wt. of embryos were suspended in 1875ml of hatch medium without antibiotics (Warner *et al.*, 1979) and incubated for 15h at 28°C with vigorous aeration.

Using these conditions less than 5% of the embryos emerge before harvesting. Dormant embryos were prepared exactly as developing embryos except incubation was in an ice bath in the absence of aeration.

Purification of *Artemia* tubulin

Preparation of S_1 . *Artemia* embryos were harvested by suction, washed with cold water followed by Pipes buffer (100mM-Pipes/1mM-MgCl₂/1mM-EGTA/0.1mM-GTP, pH6.9 at 20°C) containing 4M-glycerol and homogenized in a mortar and pestle with 15ml of Pipes buffer containing 4M-glycerol. The homogenate was stirred for 20min at 4°C, centrifuged at 16000g for 10min at 4°C and the resulting supernatant, after passage through glass wool, was centrifuged at 40000g for 30min. The 40000g supernatant (S_1) was frozen at -70°C.

Phosphocellulose (P11) chromatography. The S_1 (450–500mg of protein) was applied at a flow rate of 40ml/h to a column (19cm × 2.5cm) of P11 previously washed with Pipes buffer containing 1M-NaCl and equilibrated with Pipes buffer. The protein that did not adhere to the column was collected as 4ml samples which were pooled and frozen at -70°C.

DEAE-cellulose chromatography. The P11 fraction (225–280mg of protein) was applied to a column (18cm × 2.5cm) of DEAE-cellulose which was previously washed with Pipes buffer containing 1M-NaCl and equilibrated with Pipes buffer. The protein was eluted sequentially at a flow rate of 60ml/h with Pipes buffer, Pipes buffer containing 0.2M-NaCl and Pipes buffer containing 0.3M-NaCl. The fractions from the final wash contained tubulin and these were pooled.

(NH₄)₂SO₄ fractionation. The 0.3M-NaCl fraction from DEAE-cellulose was immediately brought to 50% saturation (29.1g/100ml) with (NH₄)₂SO₄. After stirring for 20min at 4°C the precipitate was collected by centrifugation, resuspended in 1.0ml of Pipes buffer and dialysed overnight against 250ml of Pipes buffer containing 8M-glycerol.

Assembly-disassembly of microtubules. After dialysis the protein preparation was diluted 1:1 with Pipes buffer and centrifuged at 40000g for 30min. The concentrations of GTP and MgCl₂ were brought to 1.9mM and 11mM respectively and the solution was incubated at 35°C for 20–30min to allow microtubule assembly. The microtubules were collected by centrifugation at 40000g for 30min, resuspended in 0.3ml of cold Pipes buffer, incubated at 4°C for 30–40min and centrifuged twice at 40000g for 30min. The final 40000g supernatant which contained the purified tubulin was stored at -70°C.

During purification, the protein concentration

was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Tubulin purity was monitored by SDS/polyacrylamide-gel electrophoresis.

Preparation of bovine brain tubulin

Microtubule proteins used for all procedures except peptide mapping were purified from fresh bovine brain by a modification of the Shelanski *et al.* (1973) method of assembly-disassembly. The brain was homogenized in Pipes buffer containing 4M-glycerol and the microtubule proteins were cycled two times with glycerol present only for the first assembly. The cycled microtubule proteins were stored at -70°C . To obtain homogeneous tubulin, a sample of the twice-cycled microtubule proteins was thawed, cycled once and applied to a column of P11 previously washed with Pipes buffer containing 1M-NaCl and equilibrated with Pipes buffer. The tubulin, which did not adhere to P11 under these conditions, was collected, dialysed overnight against Pipes buffer containing 8M-glycerol, diluted 1:1 with Pipes buffer and recycled to yield homogeneous bovine brain tubulin. The bovine tubulin was stored at -70°C in the absence of glycerol.

Gel analysis of tubulin

Electrophoresis in 7.5% polyacrylamide gels ($0.5\text{cm} \times 10\text{cm}$) containing 0.1% SDS was performed at 8 mA/gel in 0.1M-phosphate buffer at pH 7.2 essentially as described by Weber & Osborn (1969). Slab gel electrophoresis was performed in 7.5% polyacrylamide gels containing 0.13% SDS at 10mA/gel essentially as described by Laemmli (1970). The gels measured $11\text{cm} \times 16\text{cm} \times 0.75\text{mm}$.

Isoelectric focusing of tubulin solubilized in 4.5M-urea was performed essentially as described by Dahl & Weibel (1979) with some modifications. A gradient of pH 6.9 to 5.0 was established in a 4% polyacrylamide gel support using a 4:1 mixture of Bio-Lyte 5/7 and 3/10 ampholytes (BioRad Laboratories). After polymerization, the gels, with dimensions of $0.3\text{cm} \times 11\text{cm}$, were prefocused for 15 min at 200 V, 30 min at 300 V and 30 min at 400 V. Samples were applied to the basic end of the gel and electrophoresis continued for 15 h at 400 V and 1 h at 800 V.

Gels, fixed in a solution containing 4% (w/v) sulphosalicylic acid and 12.5% (w/v) trichloroacetic acid, were stained with 0.04% Coomassie Blue R250 dissolved in a solution containing 27% (v/v) propan-2-ol, 10% (v/v) acetic acid and 0.5% CuSO_4 . Destaining was in a 12% (v/v) solution of propan-2-ol containing 7% (v/v) acetic acid and 0.5% CuSO_4 followed by a solution of 7% (v/v) acetic acid and 5% (v/v) methanol.

The pH profile of isoelectric focusing gels was determined by placing 1 cm sections of blank gels, electrophoresed in parallel with sample gels, in 1 ml of 0.025M-KCl for 3 h at room temperature prior to measurement of the pH.

Second dimension analysis of isotubulins separated by isoelectric focusing was done at 25mA/gel on SDS/polyacrylamide-slab gels ($11\text{cm} \times 16\text{cm} \times 1.5\text{mm}$) essentially as described above.

Peptide mapping

Tubulin was isolated from the cerebellum of bovine brains, purchased from the Roegelein Co., San Antonio, TX, U.S.A., by one cycle of assembly-disassembly followed by phosphocellulose chromatography (Fellous *et al.*, 1977).

Artemia and bovine tubulins were reduced and carboxymethylated (Crestfield *et al.*, 1963) followed by electrophoresis on 6% polyacrylamide-slab gels (Laemmli, 1970). The α - and β -tubulin subunits, visualized by soaking the slab gel in 4M-sodium acetate (Higgins & Dahmus, 1979) were removed and recovered by elution. The purified α - and β -subunits were digested with V-8 proteinase from *Staphylococcus aureus* and electrophoresed on 15% polyacrylamide gels (Laemmli, 1970) according to the method of Cleveland *et al.* (1977). The gels were stained with silver (Oakley *et al.*, 1980).

Microtubule assembly assay

Artemia tubulin at a final concentration of 1.1mg/ml and bovine tubulin at 1.5–2.0mg/ml were assembled into microtubules in a $50\mu\text{l}$ assembly mixture containing 100mM-Pipes (pH 6.9), 1mM- MgCl_2 , 1mM-EGTA and 1.9mM-GTP. The MgCl_2 was increased to 11mM when glycerol, at a final concentration of 2.5M, was present. Following incubation at 35°C for 15 min, assembly was halted by addition of an equal volume of 4% glutaraldehyde in Pipes buffer at 35°C . Samples were placed on carbon-stabilized, Formvar-coated grids, stained with a solution of 1% uranyl acetate containing 0.1% cytochrome *c* and examined in a Philips 201 electron microscope.

Results

Purification of Artemia tubulin

Results for purification of tubulin from whole cell homogenates of developing and dormant *Artemia* embryos, by the procedures described in the Experimental section, are shown in Fig. 1 and Tables 1 and 2. Assessment of tubulin purity was achieved by electrophoresis of protein samples on SDS/polyacrylamide gels (Fig. 1). Following chromatography on DEAE-cellulose the *Artemia* tubulin preparation contained a prominent polypeptide which comigrated with bovine tubulin,

Fig. 1. SDS/polyacrylamide tube gel electrophoresis of tubulin at various stages of purification from developing *Artemia* embryos

Electrophoresis, by a modification of the Weber & Osborn (1969) technique, was as described in the Experimental section. (a) 100 µg of whole embryo homogenate (fraction S_1); (b) 100 µg of the flow-through from P11 (fraction P11); (c) 105 µg of the fraction eluted from DEAE-cellulose with Pipes buffer containing 300 mM-NaCl; (d) 80 µg of *Artemia* tubulin obtained after $(\text{NH}_4)_2\text{SO}_4$ fractionation and assembly-disassembly; (e) 8 µg of bovine tubulin.

as well as a number of minor bands of varying molecular weight (Fig. 1c). As shown on a heavily overloaded gel (Fig. 1d) all contaminating polypeptides were removed by $(\text{NH}_4)_2\text{SO}_4$ fractionation and assembly-disassembly during the final purification steps.

The final yield of tubulin from 125 g of developing embryos was 1 mg (Table 1), whereas 0.8 mg of protein was obtained from dormant embryos (Table 2). The yields of tubulin from dormant embryos tended to fluctuate more than from developing embryos, but approximately equal quantities of

tubulin could be obtained from embryos at both stages of development.

Microtubule assembly

The observation that *Artemia* microtubules assembled in the presence of glycerol and Mg^{2+} at the final stage of purification were abnormal

(similar in appearance to the microtubules of Figs. 2c and 2d) suggested that assembly conditions for *Artemia* and bovine brain tubulin may differ. The suggestion was verified when it was found that *Artemia* tubulin, from either developing or dormant embryos, assembled into long, structurally normal microtubules in the absence of glycerol, Mg^{2+} or MAPs (Figs. 2a and 2b), whereas the in-

Table 1. Purification of tubulin from developing *Artemia* embryos

Tubulin was purified from 125g of developing *Artemia* embryos as described in the Experimental section. The fraction termed tubulin results from subsequent purification of the P11 fraction by DEAE-cellulose chromatography, $(NH_4)_2SO_4$ fractionation and assembly-disassembly of microtubules.

Fraction	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Protein (%)
S ₁	16.7	30.0	500	100
P11	107.0	2.5	268	53
Tubulin	0.3	3.4	1	0.2

Table 2. Purification of tubulin from dormant *Artemia* embryos

Tubulin was purified from 125g of dormant *Artemia* embryos as described in the Experimental section. The fraction termed tubulin results from subsequent purification of the P11 fraction by DEAE-cellulose chromatography, $(NH_4)_2SO_4$ fractionation and assembly-disassembly of microtubules.

Fraction	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Protein (%)
S ₁	14.7	34.0	500	100
P11	104.0	2.7	281	56
Tubulin	0.3	2.7	0.8	0.2

Fig. 2. Electron micrographs of negatively stained *Artemia* microtubules assembled in vitro (a, b) *Artemia* microtubules assembled in the absence of glycerol, Mg^{2+} and MAPs; (c, d) *Artemia* microtubules assembled in the presence of 2.5M-glycerol and 11 mM- Mg^{2+} . The microtubules shown in these pictures were formed with tubulin from developing embryos. Morphologically similar microtubules were obtained with tubulin from dormant embryos and are not shown.

clusion of 2.5M-glycerol and 11 mM-Mg²⁺ resulted in short structures which appeared to be partially formed microtubules (Figs. 2c and 2d). The assembly of bovine tubulin into morphologically normal microtubules, in the buffer conditions employed for polymerization of *Artemia* tubulin, depended on MAPs or glycerol. This difference in assembly characteristics indicates a fundamental molecular difference between *Artemia* and bovine tubulin.

Slab gel electrophoresis of tubulin

The tubulin subunits from developing and dormant *Artemia* embryos comigrated and were well resolved on SDS/polyacrylamide slab gels buffered with Tris/glycine (Fig. 3). In contrast, bovine α -

and β -tubulins were less well resolved with this particular gel system and neither subunit comigrated with the corresponding *Artemia* tubulin.

Isoelectric focusing of tubulin

Upon isoelectric focusing, *Artemia* tubulin from developing and dormant embryos yielded identical banding patterns, with each being resolved into four major and several minor isoelectric variants in the pH range 5.6–6.0 (Fig. 4). Isoelectric variants of bovine tubulin, on the other hand, were not as well resolved with this system and they tended to focus at a more acidic location (pH 5.5–5.7) in the pH gradient than did the *Artemia* isotubulins. These results show that *Artemia* and bovine tubulins are clearly composed of different isoelectric variants and that during the course of pre-emergence development the isotubulin population of *Artemia* does not change.

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis of *Artemia* tubulin showed that the two major, more basic isotubulins obtained upon isoelectric focusing were α -tubulins whereas the two major, more acidic isotubulins were β -tubulins (Figs. 5 and 6). Definitive identification of all the minor isotubulins by this method was not possible, although some isoforms can be assigned the correct designation. Again, as for other electrophoretic results, the tubulins from developing and dormant embryos are identical, indicating that there is no change in tubulin during pre-emergence development of *Artemia* embryos.

Peptide mapping

The peptide maps of α - and β -tubulin from bovine brain and developing *Artemia* embryos are shown in Fig. 7. The α -tubulin maps of the two organisms are identical except that *Artemia* α -tubulin has an extra peptide. Fig. 7 also shows that the *Artemia* β -tubulin map is very similar to that of bovine brain β -tubulin, consistent with the apparent high conservation of β -tubulins generally observed. The overall result, unexpected in view of the phylogenetic distance between these two organisms and the previous data given in this paper, is that bovine and *Artemia* tubulins have markedly similar peptide maps.

Discussion

The developmental modification of tubulin, the role of MAPs and other factors in microtubule assembly and the phylogenetic interrelationships of tubulin from various organisms were examined using the brine shrimp, *Artemia*, as a source of tubulin. *Artemia* was chosen for this study as at least part of its early, relatively synchronous em-

Fig. 3. SDS/polyacrylamide-slab gel of *Artemia* and bovine tubulin

Electrophoresis, by a modification of the Laemmli (1970) procedure, was as described in the Experimental section. (a) Bovine tubulin; (b) *Artemia* tubulin from developing embryos; (c) *Artemia* tubulin from dormant embryos.

Fig. 4. Isoelectric focusing of *Artemia* and bovine tubulin

Isoelectric focusing was performed as described in the Experimental section. (a) 40 μ g of bovine brain tubulin; (b) 40 μ g of tubulin from developing *Artemia* embryos; (c) 40 μ g of tubulin from dormant *Artemia* embryos; (d, e, f) enlarged portions of gels (a), (b) and (c) respectively. The identities of *Artemia* α - and β -tubulins, as revealed by two-dimensional gel electrophoresis, are indicated.

bryo development occurs in the absence of cell division, which is a microtubule-dependent event. Thus, changes in tubulin required for development *per se* are not confused with changes which may be needed for cell division. Of somewhat more practical importance, large quantities of *Artemia* embryos are readily available, this being particularly advantageous for the study of non-neural tubulins which are usually present in small amounts in cells.

As initial attempts to assemble microtubules in cell-free homogenates of *Artemia* were unsuccessful, an alternative scheme, based on the chromatographic behaviour of tubulin from other organisms, was developed. SDS/polyacrylamide-gel

electrophoresis revealed that after DEAE-cellulose chromatography, the *Artemia* tubulin preparation contained a major polypeptide that comigrated with bovine tubulin and this was the only band on heavily overloaded gels after purification was completed. Extensive assembly of *Artemia* microtubules occurred only at the final stage of purification unless the drug, taxol (Schiff *et al.*, 1979; Vallee, 1982), which enhances microtubule formation by lowering the critical concentration for assembly, was included in the assembly mixture. This observation suggests that assembly in crude cell-free homogenates did not occur as the tubulin concentration was too low, although the presence of an inhibitor of assembly cannot be excluded.

Fig. 5. Two-dimensional gel electrophoresis of tubulin from developing *Artemia* embryos

Two-dimensional gel electrophoresis was performed as described in the Experimental section. (a) An entire slab gel with an isoelectric focusing gel attached; (b) an enlargement of the tubulin spots from (a). The identity of the major isoelectric focusing bands as α - and β -tubulin (Fig. 4) and the similarity of tubulin from developing and dormant embryos (see Fig. 6) are apparent.

Lack of microtubule assembly in extracts of non-neural cells is common and is generally attributed to low tubulin concentrations and/or inhibitors of microtubule assembly (Roobol *et al.*, 1980*a,b*; Liebes *et al.*, 1980; Kilmartin, 1981; Morejohn & Fosket, 1982; Weinert *et al.*, 1982; Detrich & Wilson, 1983; Suprenant & Rebhun, 1983).

Artemia tubulin, in contrast with bovine brain tubulin, assembles into morphologically normal microtubules in the absence of glycerol, Mg^{2+} , high molecular weight MAPs and tau proteins. Assembly of *Artemia* tubulin into short microtubule-

like structures may result from a more efficient initiation of tubulin polymerization in the presence than in the absence of glycerol. Not explained, however, is why many of the *Artemia* microtubules are incompletely formed when glycerol and Mg^{2+} are present nor why *Artemia* tubulin assembles well in the absence of MAPs or glycerol. Tubulin from several other sources, most of which are non-neural (Kuriyama, 1977; Farrell & Wilson, 1978; Keller & Rebhun, 1982; Suprenant & Rebhun, 1983; Detrich & Wilson, 1983) assembles readily at low critical concentrations in the absence of accessory

Fig. 6. Two-dimensional gel electrophoresis of tubulin from dormant *Artemia* embryos. Two-dimensional gel electrophoresis was performed as described in the Experimental section. (a) An entire slab gel; (b) an enlargement of the tubulin spots from (a). The identity of the major isoelectric focusing bands as α - and β -tubulin (Fig. 4) and the similarity of tubulin from developing and dormant embryos (see Fig. 5) are apparent.

factors. The results show that the information required for microtubule assembly is inherent in tubulin from several organisms, including *Artemia*. Questions are raised concerning the necessity and/or role of MAPs in microtubule assembly and the molecular characteristics of tubulin which determine microtubule assembly.

The pattern of major bands on the peptide maps demonstrates that *Artemia* α -tubulin is a metazoan, cytoplasmic tubulin and is not axonemal or non-animal in origin (Little *et al.*, 1981, 1982a). This finding is consistent with expected results as the purification procedure essentially precludes isolation of axonemal tubulin. It is, however, difficult to decide which other metazoan cytoplasmic tubulin most closely resembles *Artemia* tubulin. On the one

hand, the extra peptide present in *Artemia* is found in the map of α -tubulin from bovine brain or sea urchin eggs (Little *et al.*, 1981). Since the squid, a mollusc, and *Artemia*, an arthropod, are both protostomes, it is not surprising that there should be a resemblance. The remainder of the *Artemia* α -tubulin map is, however, identical with that of bovine brain α -tubulin and is more similar to bovine brain than to squid or sea urchin egg α -tubulin. The previously published map of *Drosophila* egg α -tubulin indicates that it differs substantially from other metazoan α -tubulins (Little *et al.*, 1982b) but, as *Drosophila* is an arthropod, it is somewhat surprising that its α -tubulin map is so different from that of *Artemia*. Fig. 7 also shows that the β -tubulin map of *Artemia* is virtually

Fig. 7. Peptide mapping of bovine and *Artemia* tubulin

Peptide mapping was performed as described in the Experimental section. (a) and (b) are the peptide maps of *Artemia* and bovine α -tubulin respectively; (c) and (d) are the peptide maps of bovine and *Artemia* β -tubulin respectively. The arrowheads indicate peptides found in the *Artemia* but not in the bovine maps, with the variance in the β -tubulin maps possibly dependent on slightly different extents of tubulin digestion.

identical with that of bovine brain β -tubulin, consistent with the apparent high conservation of β -tubulin reported by Little *et al.* (1981).

Due to the nonidentical gel banding patterns on

SDS/polyacrylamide gels (Fig. 3), the presence of different isotubulins (Figs. 4 and 5) and the differences in assembly characteristics, the marked similarity of the peptide maps for *Artemia* and bovine

tubulin was not expected. This finding throws a new light on the hypothesis (Little *et al.*, 1982b) that metazoan axonemal tubulins are more highly conserved in evolution than are metazoan cytoplasmic tubulins due to constraints imposed on tubulins required in formation of the highly organized axoneme. One of the cornerstones of this argument is that axonemal α -tubulin maps of organisms, phylogenetically very distant, such as the sea urchin, a deuterostome, and the clam, a protostome, are identical, whereas substantial differences were observed in cytoplasmic α -maps between deuterostomes and protostomes. In fact, the two protostome cytoplasmic α -tubulin maps previously obtained for squid and *Drosophila* had little resemblance to each other or to any deuterostome cytoplasmic α -tubulin.

The similarity of the *Artemia* and bovine α -tubulin maps suggests that there may be more than one form of cytoplasmic tubulin. One of these forms, found in *Artemia* and bovine brain, could be highly conserved in evolution, whereas the tubulins of squid brain and *Drosophila* egg may correspond to other forms of cytoplasmic tubulin. Exactly what factors may be constraining the evolution of the tubulin found in *Artemia* and bovine brain cannot presently be determined.

Isoelectric focusing and two-dimensional gel electrophoresis have been used to resolve *Artemia* tubulin into several isoforms which are identical in dormant and developing embryos but which differ from bovine brain tubulin. These data, combined with data on tubulin yields during purification, indicate that there is little change in the amount or type of tubulin in pre-emergence embryos, although quantification must be more rigorous before final conclusions can be drawn. The absence of change in tubulin isoforms during development is unusual and is worthy of extended study in relation to tubulin gene organization and expression in *Artemia* embryos. Microheterogeneity of tubulin from several organisms has been observed and is generally indicative of tubulin multi-gene families (Alexandraki & Ruderman, 1981; Mischke & Pardue, 1982; Wilde *et al.*, 1982b; Hall *et al.*, 1983; Lopata *et al.*, 1983; Thomashow *et al.*, 1983) although post-transcriptional (Wallach *et al.*, 1982; Darnall, 1982) post-translational (Brown *et al.*, 1982; Brunke *et al.*, 1982a,b; McKeithan *et al.*, 1983) or artifactual modification may generate microheterogeneity. Work is in progress to determine how the various tubulin isoforms are generated in *Artemia* and whether the type of tubulin present can be related to tubulin function during embryo development.

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References

- Alexandraki, D. & Ruderman, J. V. (1981) *Mol. Cell Biol.* **1**, 1125–1137
- Bibring, T., Baxandall, J., Denslow, S. & Walker, B. (1976) *J. Cell Biol.* **69**, 301–312
- Brown, B. A., Nixon, R. A. & Marotta, C. A. (1982) *J. Cell Biol.* **94**, 159–164
- Brunke, K. J., Collis, P. S. & Weeks, D. P. (1982a) *Nature (London)* **297**, 516–518
- Brunke, K. J., Young, E. E., Buchbinder, B. U. & Weeks, D. P. (1982b) *Nucleic Acids Res.* **10**, 1295–1310
- Cappuccinelli, P. & Morris, N. R. (eds.) (1982) *Microtubules in Microorganisms*, Marcel Dekker, New York
- Cleveland, D. & Kirschner, M. (1982) *Cold Spring Harbor Symp. Quant. Biol.* **46**, 171–183
- Cleveland, D. W., Fischer, S. G., Kirschner, M. W. & Laemmli, U. K. (1977) *J. Biol. Chem.* **252**, 1102–1106
- Cleveland, D. W., Lopata, M. A., Sherline, P. & Kirschner, M. W. (1981) *Cell* **25**, 537–546
- Crestfield, A. M., Moore, S. & Stein, W. H. (1963) *J. Biol. Chem.* **238**, 622–627
- Dahl, J. L. & Weibel, V. J. (1979) *Biochem. Biophys. Res. Commun.* **86**, 822–828
- Darnell, J. E., Jr. (1982) *Nature (London)* **297**, 365–370
- Denoulet, P., Edde, B., Jeantet, C. & Gros, F. (1982a) *Biochimie* **64**, 165–172
- Denoulet, P., Jeantet, C. & Gros, F. (1982b) *Biochem. Biophys. Res. Commun.* **105**, 806–813
- Detrich, H. W., III & Wilson, L. (1983) *Biochemistry* **22**, 2453–2462
- Doenges, K. H., Weissinger, M., Fritzsche, R. & Schroeter, D. (1979) *Biochemistry* **18**, 1698–1702
- Edde, B., Jeantet, C. & Gross, F. (1981) *Biochem. Biophys. Res. Commun.* **103**, 1035–1043
- Farrell, K. W. & Wilson, L. (1978) *J. Mol. Biol.* **121**, 393–410
- Fellous, A., Francon, J., Lennon, A.-M. & Nunez, J. (1977) *Eur. J. Biochem.* **78**, 167–174
- Ginzburg, I., Behar, L., Givol, D. & Littauer, U. Z. (1981) *Nucleic Acids Res.* **9**, 2691–2697
- Golub, A. & Clegg, J. S. (1969) *Dev. Biol.* **17**, 644–656
- Hall, J. L., Dudley, L., Dobner, P. R., Lewis, S. A. & Cowan, N. J. (1983) *Mol. Cell Biol.* **3**, 854–862
- Hentschel, C. C. & Tata, J. R. (1976) *Trends Biochem. Sci.* **1**, 97–100
- Higgins, R. C. & Dahmus, M. E. (1979) *Anal. Biochem.* **93**, 257–260
- Kalfayan, L. & Wensink, P. C. (1982) *Cell* **29**, 91–98
- Keller, T. C. S., III & Rebhun, L. I. (1982) *J. Cell Biol.* **93**, 788–796
- Kemphues, K. J., Raff, E. C., Raff, R. A. & Kaufman, T. C. (1980) *Cell* **21**, 445–451
- Kemphues, K. J., Kaufman, T. C., Raff, R. A. & Raff, E. C. (1982) *Cell* **31**, 655–670
- Kilmartin, J. V. (1981) *Biochemistry* **20**, 3629–3633
- Kuriyama, R. (1977) *J. Biochem. (Tokyo)* **81**, 1115–1125
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–682
- Lai, E. Y., Walsh, C., Wardell, D. & Fulton, C. (1979) *Cell* **17**, 867–878

- Liebes, L. F., Fleit, H., Zucker-Franklin, D. & Silber, R. (1980) *Biochim. Biophys. Acta* **633**, 245–257
- Little, M., Ludueña, R. F., Langford, G. M., Asnes, C. F. & Farrell, K. (1981) *J. Mol. Biol.* **149**, 95–107
- Little, M., Ludueña, R. F., Keenan, R. & Asner, C. F. (1982a) *J. Mol. Evol.* **19**, 80–86
- Little, M., Krauhs, E., Ponstingl, H., Ludueña, R. F. & Rice, N. (1982b) in *Microtubules in Microorganisms* (Cappuccinelli, P. & Morris, N. R., eds.), pp. 1–14, Marcel Dekker, New York
- Lopata, M. A., Havercroft, J. C., Chow, L. T. & Cleveland, D. W. (1983) *Cell* **32**, 713–724
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Ludueña, R. F. & Little, M. (1981) *Biosystems* **14**, 231–238
- McKeithan, T. W. & Rosenbaum, J. L. (1981) *J. Cell Biol.* **91**, 352–360
- McKeithan, T. W., Lefebvre, P. A., Silflow, C. D. & Rosenbaum, J. L. (1983) *J. Cell Biol.* **96**, 1056–1063
- Mischke, D. & Pardue, M. L. (1982) *J. Mol. Biol.* **156**, 449–466
- Morejohn, L. C. & Fosket, D. E. (1982) *Nature (London)* **297**, 426–428
- Neff, N. F., Thomas, J. H., Grisafi, P. & Botstein, D. (1983) *Cell* **33**, 211–219
- Oakley, B. R., Kirsch, D. R. & Morris, N. R. (1980) *Anal. Biochem.* **105**, 361–363
- Olson, C. S. & Clegg, J. S. (1978) *Wilhelm Roux's Arch. Dev. Biol.* **184**, 1–13
- Persoon, G., Sorgeloos, P., Roels, O. & Jaspers, E. (eds.) (1980) *The Brine Shrimp Artemia*, Universa Press, Wetteren
- Ponstingl, H., Krauhs, E., Little, M., Kempf, T., Hofer-Warbinek, R. & Ade, W. (1982) *Cold Spring Harbor Symp. Quant. Biol.* **46**, 191–197
- Raff, E. C. & Raff, R. A. (1978) *Am. Zool.* **18**, 237–251
- Raff, E. C., Fuller, M. T., Kaufman, T. C., Kempf, T., K. J., Rudolph, J. E. & Raff, R. A. (1982) *Cell* **28**, 33–40
- Roberts, K. & Hyams, J. S. (eds.) (1979) *Microtubules*, Academic Press, New York
- Roobol, A., Pogson, C. I. & Gull, K. (1980a) *Biochem. J.* **189**, 305–312
- Roobol, A., Pogson, C. I. & Gull, K. (1980b) *Exp. Cell Res.* **130**, 203–215
- Schiff, P. B., Fant, J. & Horwitz, S. B. (1979) *Nature (London)* **277**, 665–667
- Shelanski, M. L., Gaskin, F. & Cantor, C. R. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 765–768
- Silflow, C. D., Lefebvre, P. A., McKeithan, T. W., Schloss, J. A., Keller, L. R. & Rosenbaum, J. L. (1982) *Cold Spring Harbor Symp. Quant. Biol.* **46**, 157–169
- Spiegelman, B. M. & Farmer, S. R. (1982) *Cell* **29**, 53–60
- Stephens, R. E. (1981) *Biochemistry* **20**, 4716–4723
- Suprenant, K. A. & Rebhun, L. I. (1983) *J. Biol. Chem.* **258**, 4518–4525
- Thomashow, L. S., Milhausen, M., Rutter, W. J. & Agabian, N. (1983) *Cell* **32**, 35–43
- Valenzuela, P., Quiroga, M., Zaldivar, J., Rutter, W. J., Kirschner, M. W. & Cleveland, D. W. (1981) *Nature (London)* **289**, 650–655
- Vallee, R. B. (1982) *J. Cell Biol.* **92**, 435–442
- Vallee, R. B. & Davis, S. E. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1342–1346
- Wallach, M., Fong, D. & Chang, K.-P. (1982) *Nature (London)* **299**, 650–652
- Warner, A. H., MacRae, T. H. & Wahba, A. J. (1979) *Methods Enzymol.* **60**, 298–311
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
- Weinert, T., Cappuccinelli, P. & Wiche, G. (1982) *Biochemistry* **21**, 782–789
- Wilde, C. D., Chow, L. T., Wefald, F. C. & Cowan, N. J. (1982a) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 96–100
- Wilde, C. D., Crowther, C. E. & Cowan, N. J. (1982b) *J. Mol. Biol.* **155**, 533–538
- Wilde, C. D., Crowther, C. E., Cripe, T. P., Lee, M. G.-S. & Cowan, N. J. (1982c) *Nature (London)* **297**, 83–84