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The synthesis of tubulin and its post-translational modification during development of the brine shrimp, *Artemia*

by

Carrie Mae Langdon

Submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy

at

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Abstract

Development in most organisms is accompanied by changes in their tubulin composition, commonly due to differential gene expression and/or post-translational modifications. The brine shrimp, *Artemia*, undergoes a period of post gastrular morphogenesis in the absence of mitosis, resulting in the formation of a free-swimming nauplius. Two-dimensional gel electrophoresis revealed that the isotubulin family, composed of three α- and two β-tubulins, does not change during development of *Artemia franciscana*. The generation of isotubulin diversity in this unusual organism was investigated. One size class each of α- and β-tubulin mRNA, 1.9 kB in length, was detected on Northern blots using cloned *Drosophila* tubulin gene probes. Only a small amount of the tubulin mRNA was present on polysomes. The amount of tubulin mRNA on polysomes or in the cytoplasm did not change over development. *In vitro* translation of *Artemia* poly(A)$^+$ RNA yielded one α- and a maximum of two β-tubulins on two-dimensional gels; total cytoplasmic RNA gave a larger amount of tubulin synthesized *in vitro* than did polysomal RNA. *In vivo* labelling of *Artemia* proteins showed a low level of tubulin synthesis. It appears that there is translational regulation of *Artemia* tubulin synthesis during early post-gastrular development. Analysis of post-translational modifications to *Artemia* tubulin revealed the presence of acetylated and tyrosinated α-tubulin; acetylated α-tubulin localized to only one of the α-isotubulin spots on two-dimensional gels. Isotubulin diversity in *Artemia* is provided at least in part by post-translational modifications. Detyrosinated α-tubulin was absent until 20 h of development had passed, which demonstrates that this tubulin is not required for early development in a complex, metazoan animal. Evidently a small number of tubulins can carry out the numerous microtubule activities in a multicellular organism, supporting the theory that tubulins are multifunctional.

LIST OF ABBREVIATIONS

ATP	adenosine 5'-triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
DEPC	diethylpyrocarbonate
DMSO	dimethylsulfoxide
DTT	dithiothreitol
EDTA	ethylenediaminotetraacetic acid
EGTA	ethylene glycol-bis(ß-aminoethyl ether)-N,N,N',N' tetraacetic acid
Glu	detyrosinated
GTP	guanosine 5'-triphosphate
HST	high salt TBS-Tween
IEF	isoelectric focusing
kB	kilobases
kD	kilodaltons
LMW	low molecular weight markers
MOPS	3-(N-morpholino)propanesulfonic acid
NaOAc	sodium acetate
NaOH	sodium hydroxide
NBT	nitroblue tetrazolium
NP-40	Nonidet-P40
Pipes	1,4-piperazinediethanesulfonic acid
PMSF	phenylmethylsulfonyl fluoride
SDS	sodium dodecyl sulfate
TBS	Tris buffered saline

TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TEMN	Tris-EDTA-$MgCl_2$-NaCl buffer
TMES	Tris-$MgCl_2$-EDTA-sucrose buffer
Tris	tris-(hydroxymethyl)aminomethane
Tween	polyoxyethylene sorbitan monolaurate
Tyr	tyrosinated

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INTRODUCTION

Tubulin, the major component of microtubules, is composed of two globular polypeptides of approximately 450 amino acids each, α and β, which have related amino acid sequences, and a combined molecular weight of approximately 100 kD. Both α- and β-tubulins show regions of sequence variability, particularly at the carboxy terminus, interspersed within a fairly well-conserved framework. Recently a third isotubulin, γ-tubulin, was found, but it has only been detected in *Aspergillus nidulans* (Oakley and Oakley, 1989). The α- and β-isotubulins form α/β dimers, which have the ability to polymerize into microtubules: cylindrical organelles 25 nm in diameter which function in numerous cellular activities such as cell division, maintenance of intracellular architecture, cell motility, vesicle transport, and differentiation (Cooper *et al.*, 1990; Jasmin *et al.*, 1990; Kelly, 1990; Matthews *et al.*, 1990; Gundersen *et al.*, 1989; Wadsworth *et al.*, 1989; Lai *et al.*, 1988).

In most eukaryotes, multiple α- and β-tubulin genes exist, and tubulin pseudogenes may also be present. Generally the tubulin genes are dispersed throughout the genome, but in *Trypanosoma cruzi* α- and β-tubulin mRNAs originate from a single, multicistronic transcript of alternating α- and β-tubulin mRNAs (Soares *et al.*, 1989; Maingon *et al.*, 1988) which is the product of a repeated series of linked tubulin genes. In a very different organism, *Zea mays*, an alternat-

ing arrangement of tubulin genes has also been revealed, but in this case the genes are for two different α-tubulins (Montoliu *et al.*, 1989). In most organisms, tubulin gene expression is spatially and temporally regulated. Mouse, chicken, and *Drosophila* tubulin gene expression have been particularly well documented (MacRae and Langdon, 1989). Evidence of differential tubulin gene expression led Fulton and Simpson (1976) to propose that multiple tubulin genes exist because the tubulins they encode are functionally specialized to perform particular activities in cells. The function of a microtubule would therefore be determined by its isotubulin composition. Under this scheme, the functional specificity of a particular tubulin is indicated by the regions of amino acid sequence variability within the molecule. Raff has offered an alternate explanation: all isotubulins are functionally equivalent, and multiple tubulin genes are present to permit transcriptional regulation of tubulin synthesis (Raff *et al.*, 1987; Raff, 1984), presumably via upstream regulatory sequences and perhaps other cell-specific factors. Regions of sequence variability would not indicate functional specificity, but rather would indicate regions where sequence divergence, as a result of genetic drift, can be tolerated. Conserved regions, on the other hand, are those whose sequences are essential for the functioning of the tubulin molecule (Lopata and Cleveland, 1987; Raff *et al.*, 1987; Raff, 1984), such as microtubule-

associated protein binding sites, calcium and GTP binding sites, and areas of α/β tubulin interaction. The latter proposal is supported by abundant experimental evidence that very divergent or even chimeric tubulins will co-polymerize with normal tubulins in cells and become integrated into all microtubule structures (Gu *et al.*, 1988; Joshi *et al.*, 1987; Lewis *et al.*, 1987; Bond *et al.*, 1986; Swan and Solomon, 1984). Nonetheless, evidence for differential utilization of tubulin isoforms is still being uncovered (Joshi and Cleveland, 1989). In chicken erythrocytes a divergent β-tubulin has been observed which has biochemical properties and assembly characteristics distinct from other β-tubulins, and which contributes specifically to the erythrocyte cytoskeleton (Monteiro and Cleveland, 1988; Murphy *et al.*, 1987). Therefore, neither hypothesis can be definitively accepted as yet, and it seems likely that both hypotheses may prove to be correct.

Transcription is the primary level at which differential tubulin gene expression is achieved. The tubulins expressed in any particular organism may change over development, and are frequently organ and/or tissue specific (for a review, see MacRae and Langdon, 1989). Transcriptional regulation has also been demonstrated to occur in response to deflagellation and flagella development in a number of organisms. Differentiation of the unicellular eukaryote *Naegleria gruberi* from an amoeba to a flagellate is

accompanied by a dramatic increase in the level of tubulin mRNA (Lai *et al.*, 1988). *Tetrahymena*, *Chlamydomonas*, and sea urchins show a burst in tubulin gene transcription following deflagellation (Gong and Brandhorst, 1987; Seyfert, 1987; Baker *et al.*, 1986, 1984). The increase of tubulin mRNA synthesis is not affected by the size of the intracellular tubulin pool, and has been proposed to be part of a coordinated increase in transcription of a block of genes encoding flagellar components (Gong and Brandhorst, 1987; Shea and Walsh, 1987). In each of the above organisms, an increase in the stability of tubulin mRNA has also been noted, and is likely to be an important factor in the production of increased amounts of tubulin. The cellular mechanisms responsible for regulation of tubulin gene transcription are not as yet known.

Tubulin expression is not only controlled by differential transcription of the tubulin genes, but also by translational regulation of tubulin mRNAs. Cleveland and coworkers have elucidated an autoregulatory mechanism in cultured mammalian cells by which the stability of tubulin mRNA is modulated such that the synthesis of tubulin meets, but does not exceed, the demand for it (Gay *et al.*, 1989; Yen *et al.*, 1988a; Yen *et al.*, 1988b). That such regulation is important was shown in yeast, where overproduction of tubulin is lethal (Burke *et al.*, 1989). Cleveland's model involves the cotranslational binding of soluble tubulin to the amino ter-

minal tetrapeptide of a nascent tubulin polypeptide as it exits the ribosome. This binding produces transient ribosome stalling and/or a conformational change which presumably activates a ribosome-associated ribonuclease which degrades the tubulin mRNA. Clearly, only the translation of those tubulin mRNAs which are in polysomes is autoregulated, and if there is a deficit of soluble tubulin in the cytoplasm there will be no free tubulin to bind to nascent tubulins, so translation will proceed. Evidence for autoregulation of tubulin synthesis has been provided by Gong and Brandhorst (1988a, 1988b) using sea urchin embryos, making it likely that Cleveland's model not only applies to the artificial environment of cell culture, but to whole organisms as well.

In addition to differential gene expression, tubulin diversity can also arise as a result of post-translational modifications. Acetylation/deacetylation, tyrosination/detyrosination and, recently, glutamylation (Eddé *et al.*, 1990) have been found to occur on α-tubulin. Phosphorylation of α- and β-tubulin has also been documented (Ludueña *et al.*, 1988; Serrano *et al.*, 1987; Gard and Kirschner, 1985). The production of antibodies specific for acetylated, tyrosinated, and detyrosinated α-tubulin has greatly facilitated the study of these three post-translational modifications.

Acetylation is a reversible post-translational modification to α-tubulin originally characterized in *Chlamydomonas* flagella. Using the monoclonal antibody 6-11B-1, it was

found that amino acid residue 40, a lysine (L'Hernault and Rosenbaum, 1985; LeDizet and Piperno, 1987), is acetylated by a specific α-tubulin acetylase (Maruta *et al.*, 1986; Greer *et al.*, 1985) shifting the α-tubulin to a more acidic position on two-dimensional gels. Acetylation primarily occurs on polymerized microtubules (Bulinski *et al.*, 1988a; dePennart *et al.*, 1988) and acetylated microtubules tend to be resistant to drug-induced depolymerization (Khawaja *et al.*, 1988; Piperno *et al.*, 1987; LeDizet and Piperno, 1986). Although originally discovered in the stable microtubules of flagella, further research using different organisms and cell cultures showed that acetylated α-tubulin is found in microtubules of varying intrinsic stability (Webster and Borisy, 1989; Sasse and Gull, 1988; Piperno *et al.*, 1987). It appears that acetylation of α-tubulin serves - at least in some cases - as an indicator rather than an effector of microtubule stability. For example, acetylation may serve in the recognition of microtubules by stabilizing microtubule-associated proteins. During development of mouse oocytes and embryos (Houliston and Maro, 1989; dePennart *et al.*, 1988; Schatten *et al.*, 1988) and in *Physarum* (Sasse *et al.*, 1987), α-tubulin acetylation appears to be both temporally and spatially regulated, but until the physiological significance of acetylated α-tubulin is more clearly understood, conclusions regarding regulation of acetylation are difficult to draw.

The processes of tyrosination and detyrosination act in a cyclic fashion on α-tubulin (Bulinski *et al.*, 1988b). Most α-tubulins bear a carboxy terminal tyrosine, which is removed by tubulin carboxypeptidase (Wehland and Weber, 1987; Argaraña *et al.*, 1980) and is replaced by tubulin tyrosine ligase (Raybin and Flavin, 1977). Detyrosination, like acetylation, primarily occurs on α-tubulin which is assembled into microtubules (Wehland and Weber, 1987; Arce and Barra, 1983), and the association of tubulin carboxypeptidase with microtubules has been demonstrated by the co-purification of the carboxypeptidase with tubulin (Arce and Barra, 1983). Conversely, tyrosination occurs primarily on unpolymerized tubulin (Gundersen *et al.*, 1987; Arce *et al.*, 1978). In general, microtubules rich in detyrosinated α-tubulin are more stable than those rich in tyrosinated α-tubulin (Gundersen and Bulinski, 1988; Khawaja *et al.*, 1988; Bré *et al.*, 1987). The physiological significance of the tyrosination/detyrosination process is not known, but there are numerous examples of stable arrays of microtubules forming in differentiating cells which are enriched in detyrosinated α-tubulin (Sasse and Gull, 1988; Wehland and Weber, 1987; Gundersen and Bulinski, 1986). As is the case for acetylated α-tubulin, it appears that detyrosinated α-tubulin is a consequence rather than a cause of microtubule stability. Again, microtubule-associated proteins (Lee, 1990; Vallee, 1990) have been proposed to act as stabilizing

agents (Falconer *et al.*, 1989; Bulinski *et al.*, 1988a), but there is no experimental evidence to support this at present.

The brine shrimp, *Artemia*, is a crustacean of the class Branchiopoda, and is found world-wide in saline environments, such as the Great Salt Lake in Utah (Heip *et al.*, 1976). *Artemia* young may be released from the female as live, free-swimming nauplii, but under conditions of high salinity and low oxygen may instead be shed as dormant gastrulae encased in a cyst shell. Large amounts of such cysts can be collected and stored under vacuum for long periods of time without appreciable loss of viability. Inside the cyst - which is impermeable to most substances, including organic solvents (Heip *et al.*, 1976) - the *Artemia* gastrula is in a cryptobiotic state and shows no metabolic activity whatsoever. *Artemia* is one of the few known metazoan organisms able to withstand anaerobic dormancy (Busa, 1985). The mechanism(s) controlling dormancy in *Artemia* gastrulae has not been completely elucidated, but it is thought that pH could be a major regulatory factor, as the activities of the many metabolic pathways in a living organism could presumably only be coordinated by a broad spectrum effector such as pH (Busa, 1985). The dormant cyst contains 2-6% (dry weight) glycerol, which is believed to protect proteins from denaturation, oxidation, and radiation while itself being relatively non-toxic and non-volatile (Heip *et al.*, 1976). A further 17% of the dry weight of the cyst is made up of

trehalose, a non-reducing carbohydrate synthesized from glycogen at the onset of the dormancy process, and converted back to glycogen when development resumes (Heip *et al.*, 1976). Dormancy is terminated when an adequate temperature and an appropriate saline environment are provided. The organism rapidly develops from a gastrula, to a prenauplius still partly enclosed within a hatching membrane, then hatches as a swimming nauplius after approximately 17 h (Go *et al.*, in press; Rafiee *et al.*, 1986a). The newly hatched nauplius is in instar 1: it has a relatively well-developed head region, an unsegmented thorax, and does not initially have a functional digestive tract, living instead off yolk reserves. After molting approximately 16 h later, the instar 2 larva has begun to filter feed using a functional, though still developing, digestive tract (Schrehardt, 1987) and has begun to show segmentation of the thorax. In total, the larvae grow and differentiate through fifteen molts. What is most interesting is that the initial period of development from a gastrula to a swimming instar 1 nauplius is achieved in the absence of mitosis. Even when mitosis resumes, shortly after hatching (Bagshaw, 1982), only a 10% increase in the number of nuclei is seen and further, if newly hatched larvae are treated with 5-fluorodesoxyuridine to block DNA synthesis, the nauplii continue to grow and develop for three days (Heip *et al.*, 1976). Early post-gastrular development in *Artemia* is evidently achieved by cell rearrangement and

differentiation, but the precise mechanism by which these processes are achieved are not known. *Artemia* is thus an interesting model system in which to study the early development of a complex, multicellular organism separate from cell division.

In other organisms, microtubules are active components in cell differentiation, and tubulin has been demonstrated to be differentially expressed and post-translationally modified during development. It therefore seemed possible that microtubules could be playing an important role during *Artemia* development. In this laboratory studies were undertaken to determine the isotubulin composition in *Artemia franciscana*. It was found that in dormant embryos, in organisms developed for 15 h, and in instar 1 nauplii, there were three major α-tubulins and two major β-tubulins on two-dimensional gels (Rafiee *et al.*, 1986b). Further research revealed a tubulin gene family of limited complexity, having a single α-tubulin gene and a small number of β-tubulin genes (Langdon *et al.*, 1990). The present study was undertaken to examine the regulation of tubulin synthesis and the mechanisms responsible for generation of isotubulin diversity during early post-gastrular development of *Artemia franciscana*. It was found that single size classes of mRNAs for α- and β-tubulin are maintained at a constant level during early development and are translated at a low level throughout the first 24 h of development to yield one α- and a maximum of

two β-tubulins. The synthesis of tubulin, then, appears to be translationally regulated in *Artemia*. Isotubulin diversity in *Artemia* is at least partly provided by post-translational modifications. In addition, the first 15-20 h of post-gastrular development occur in the complete absence of detyrosinated α-tubulin, which is first detected in the instar 1 larval stage. It thus appears that detyrosinated α-tubulin is not required for the early growth and development of this complex, metazoan animal.

MATERIALS AND METHODS

Hydration and incubation of *Artemia*

Fifty g of dormant *Artemia franciscana* cysts (Sanders Brine Shrimp Co., Ogden, Utah) were added to approximately 400 mL of cold distilled H_2O and allowed to stand at 4 °C with occasional stirring for 6 h. Hydrated cysts were collected by suction in a Buchner funnel, rinsed several times with cold distilled H_2O, and either used immediately or stored at -20 °C. Hydrated cysts were used without incubation (0 h), or were incubated in hatch medium (Appendix 1) at 28 °C in the dark on a rotary shaker (250 rpm) for the required length of time.

Preparation of cell-free homogenates and tubulin

Artemia cell-free homogenates, *Artemia* tubulin, and bovine brain tubulin were prepared as described by MacRae and Ludueña (1984). The purified tubulins were kindly supplied by Elizabeth Campbell or Thomas MacRae.

Cell-free homogenates from organisms developed 17 h (instar 1), 20 h, 22.5 h, 33 h (instar 2), and 43 h (instar 3) were prepared in a somewhat different manner from the other cell-free homogenates. Organisms were incubated in the usual fashion for the first 15 h. After 15 h of development, the *Artemia* were poured into a round shallow dish, stirred to deposit the organisms in the center, and placed in the dark with a light shining on one side of the dish to attract the swimming nauplii. Those nauplii that had congregated by the

light were drawn off after 10 min. After a further 50 min, nauplii attracted to the light were again drawn off. One hour later, nauplii which had been attracted to the light and had thus hatched within the hour (instar 1) were collected. The first instar organisms were either homogenized immediately or were placed in a 1 L separatory funnel containing 800 mL of hatch medium. The mouth of the funnel was loosely covered, and air was bubbled through the spigot of the flask. The *Artemia* were grown in the flask for 3, 5.5, 16, or 26 h, yielding orgnanisms of 20, 22.5, 33, or 43 h of development, respectively.

Following incubation, the organisms were collected by filtration on 100 µm Nitex mesh (B. and S.H. Thompson Ltd.) and immediately homogenized by hand in 750 µL of Pipes buffer pH 6.5 (Appendix 1) containing 4 M glycerol, 10 µL of each of the proteolytic enzyme inhibitor solutions A and B (Appendix 1),10 µL of 1,10-phenanthroline from a 10 mg/mL stock, and 10 µL of hydrocinnamic acid from a 10 mg/mL stock in a 15 mL Wheaton glass homogenizer ("B" plunger). The homogenate was centrifuged at 4 °C in a Beckman JA-20 rotor as follows, with the pellet discarded after each centrifugation: 10 min, 12,000 x*g*; 30 min, 48,000 x*g*; 20 min, 48,000 x*g*. The cell-free homogenates were assayed for protein content by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard, and stored at -70 °C.

SDS-polyacrylamide gel electrophoresis

One-dimensional discontinuous SDS-polyacrylamide gel electrophoresis was carried out essentially as described by Laemmli (1970). Running gels were 10% polyacrylamide, pH 8.8, and stacking gels were 4% polyacrylamide, pH 6.8. Electrophoresis was carried out at 30 mA per gel for 3 to 4 h, after which the gels were stained with Coomassie Blue for 30 minutes and destained overnight (Appendix 1).

Isoelectric focusing

Isoelectric focusing (IEF) was carried out in 12.5 cm glass tubes with internal diameters of 3 mm. Glass tubes were soaked in a dilute solution of Photo-Flo (Kodak), dried, and sealed at one end with Parafilm. The IEF gels were 4% acrylamide, contained 9 M urea, and were 10 cm in length. A pH gradient of approximately 5 to 7 was established using Bio-Lyte 3/10 and 5/7 ampholytes (Bio-Rad) in a ratio of 4:1 respectively (Appendix 1). Before electrophoresis, gels were topped with 20 µL of overlay buffer (Appendix 1) and prefocused at 200 V for 15 min, 300 V for 30 min, and 400 V for 30 min. The tops of the gels were flushed with cathode solution (Appendix 1), topped with sample made up 1:1 in overlay buffer, and focused at 400 V for 16 h, followed by 800 V for 1 h. Gels were fixed for 3 h, stained for 3 h, destained overnight in destain I, and then for 3-5 h in fresh destain I before being placed in destain II overnight (Appendix 1). During each run, two blank gels were subjected to

electrophoresis. At the end of the run the gels were cut into 1 cm lengths, and each gel piece was placed in 1.5 mL of 25 mM KCl. After 5-7 h the pH of the KCl solution containing the gel pieces was determined, in order to obtain a pH profile for each isoelectric focusing run.

Two-dimensional gel electrophoresis

Following isoelectric focusing, IEF gels were denatured in two-dimensional gel denaturing solution (Appendix 1) for two incubations of 35 min each, the solution being changed after the first incubation. Following a very brief and gentle rinse in distilled H_2O, the denatured IEF gels were affixed to the top of a 4% stacking gel using 1% agarose in 25% stacking gel buffer. The second dimension SDS-polyacrylamide gel was prepared and electrophoresed as for one-dimensional SDS-polyacrylamide gel electrophoresis. A few drops of 1% bromophenol blue were added to the upper chamber buffer as tracking dye.

***Drosophila* tubulin gene clones**

Cloned *Drosophila* α- and β-tubulin genes were contained in plasmids pDmTα1 (Kalfayan and Wensink, 1981; Teurkark et al., 1986) and DTB2 (Natzle and McCarthy, 1984) respectively. Dr. P.C. Wensink (Dept. of Biochemistry, Rosenthal Basic Medical Sciences Research Center, Brandeis University, Waltham, Mass.) supplied pDmTα1 and Dr. J.E. Natzle (Dept. of Biochemistry and Biophysics, University of California, San

Francisco, California) supplied DTB2. A map of each plasmid showing some of their restriction sites is shown in Appendix 2.

Fragments of the *Drosophila* tubulin gene clones consisting only of tubulin coding sequences were obtained by first digesting the plasmids pDmTα1 with *Bam*HI and *Xho*I, and DTB2 with *Ava*I. Digested plasmids were electrophoresed on 1% agarose gels in 1X TAE (Appendix 1) at 25 V overnight, stained with ethidium bromide, and visualized on a UV transilluminator. Troughs were cut into the gel just ahead of the desired fragments (900 bp for pDmTα1 and 1500 bp for DTB2), filled with buffer, and the fragments electrophoresed into the troughs at 150 V. The fragments were concentrated by ethanol precipitation. Approximately 250 ng of each fragment were ^{32}P-labelled by nick translation and used to probe Northern blots.

Isolation of cytoplasmic RNA

All glassware used in the isolation of RNA was soaked in 0.1% DEPC overnight and autoclaved. SDS was made up as a 20% stock and heat-treated for 1 h at 65 °C. All solutions, except phenol and chloroform, were autoclaved; those containing SDS or DTT were initially made up without these ingredients, autoclaved, and the remaining components added after the solution had cooled. All plasticware was autoclaved, and all distilled H_2O was treated with 0.1% DEPC

prior to autoclaving. Unless otherwise noted, all operations were carried out on ice.

The method used for the isolation of cytoplasmic RNA was based on that of Timberlake *et al.* (1971), with modification by Bagshaw *et al.* (personal communication) and Rafiee (personal communication).

After incubation, *Artemia* embryos or nauplii were collected by suction filtration and rinsed with cold water. Fifteen g of *Artemia* were homogenized by hand for 12 min in a prechilled mortar and pestle with 5 mL of ice-cold TEMN buffer (Appendix 1). Twenty-five mL of ice-cold TEMN buffer were added after homogenization to give 2 mL of TEMN buffer per gram of *Artemia*. The homogenate was quickly mixed, filtered through Miracloth, and centrifuged at 12,000 x*g* for 15 min. The orange coloured lipids were removed from the top of the tube so as not to contaminate the supernatant as it was collected. The volume of the supernatant was determined and guanidine-HCl added to 6 M. The solution was then heated at 65 °C for 20 min with occasional swirling, quickly chilled to room temperature on ice, and extracted once with phenol : chloroform : isoamyl alcohol (25:24:1). The phenol contained 0.1% 8-hydroxyquinoline and had been freshly washed with TMES (Appendix 1) buffer. Phases were separated by centrifugation at 12,000 x*g* for 20 min. The aqueous layer was extracted with chloroform : isoamyl alcohol (24:1), centrifuged as above, adjusted to 0.6 M NaOAc and precipitated by the addi-

tion of at least two volumes of ice-cold ethanol followed by incubation at -20 °C overnight. Precipitated RNA was collected by centrifugation at 12,000 xg for 25 min at 0 °C. RNA pellets were dried, resuspended in a minimal volume of sterile distilled H_2O, and reprecipitated in ethanol at -20 °C overnight. The reprecipitated RNA was dried, resuspended in sterile distilled H_2O and frozen at -70 °C.

Preparation of poly(A)$^+$ RNA by oligo-dT cellulose chromatography

Poly(A)$^+$ RNA was prepared from total cytoplasmic RNA by oligo-dT cellulose chromatography. Three hundred mg of oligo-dT cellulose (Bethesda Research Laboratories) was suspended in 3 mL loading buffer (Appendix 1) and poured into a sterile 3 mL syringe which had been plugged with sterile glass wool and fitted with a length of sterile tubing and a clamp. The oligo-dT cellulose was washed and packed by sequentially passing the following through the column: 5 mL sterile distilled H_2O, 5 mL oligo-dT regenerating solution (Appendix 1), and sterile distilled H_2O until the pH of the eluant was below 8.0. The column was then washed with 10 mL of loading buffer. Total cytoplasmic RNA in sterile distilled H_2O was heated in a 65 °C bath for 5 min, diluted with an equal volume of 2X loading buffer (Appendix 1) at 65 °C, and cooled rapidly on ice to room temperature. The RNA was applied to the oligo-dT column at a flow rate of 1.5 mL/min. The flow-through was collected, heated to 65 °C for

5 min, cooled on ice to room temperature, and re-applied to the column. In total, the RNA passed through the column four times. The oligo-dT cellulose was washed with 25 mL of loading buffer, until the A_{260} of the flow-through was constant at or below 0.03. The column was washed with eluting buffer (Appendix 1) at a flow rate of 0.5 mL/min. The A_{260} of 1.5 mL fractions was determined in a Perkin-Elmer Lambda-3B spectrophotometer, and all fractions having an A_{260} of 0.25 or greater were pooled. Commonly this was the first 5 or 6 fractions. The pooled fractions were adjusted to 0.3 M NaOAc and precipitated overnight with at least 2 volumes of ice-cold ethanol at -20 °C. The precipitated RNA was collected by centrifugation at 12,000 x*g* for 20-25 min, the pellet rinsed very gently with a few mL of ice-cold ethanol, dried, and resuspended in 100 µL sterile distilled H_2O.

Preparation of polysomal RNA

The procedure for preparation of polysomal RNA was the same as that described for cytoplasmic RNA until the initial 12,000 x*g* supernatant was obtained. The 12,000 x*g* supernatant was divided into two centrifuge tubes, underlayed with 5 mL/tube of TEMN buffer containing 30% RNase-free sucrose and centrifuged at 113,000 x*g* in a Beckman SW28 swinging bucket rotor for 4 h at 4 °C. The supernatant was discarded and each clear pellet was resuspended in 5 mL of sterile TE pH 8.0 (Appendix 1) on ice. The two tubes were pooled, adjusted to 6 M guanidine-HCl, incubated at 65 °C for 20 min

with frequent swirling, and quickly cooled to room temperature on ice. The polysomal RNA preparation was then extracted and ethanol precipitated as for cytoplasmic RNA preparations. Poly(A)$^+$ polysomal RNA was prepared by oligo-dT cellulose chromatography as for cytoplasmic RNA.

Electrophoresis of RNA

A 1.5% agarose mini gel containing 6% formaldehyde was used for the electrophoretic analysis of RNA. The gel was prepared by melting 0.45 g of agarose in 22.2 ml of distilled H_2O. The molten agarose was cooled to ~50 °C; 3 mL of 10X RNA gel buffer (Appendix 1) and 4.9 mL of 37-40% formaldehyde were added to the agarose, the solution was rapidly mixed then poured. After setting for 45 min, the gel was equilibrated in 1X RNA electrophoresis buffer (Appendix 1) for 30 min. While the gel was equilibrating, the RNA samples were prepared according to Sambrook *et al.* (1989). Electrophoresis was carried out at 50 V for 4 h, or until the bromophenol blue tracking dye (Appendix 1) was at the bottom of the gel. An RNA ladder (Bethesda Research Laboratories) was run in an outside lane to provide size markers.

Characterization of tubulin mRNA on Northern blots

Following electrophoresis, RNA-formaldehyde gels were rinsed in distilled H_2O for 5 min with gentle agitation. The gels were denatured in Northern denaturing solution (Appendix 1) for 40 min with gentle agitation, neutralized (Appendix 1)

for 40 min, again with gentle shaking and soaked for 10 min in 20X SSC. RNA was transferred to nitrocellulose (Schleicher and Schull) by capillary action in 20X SSC (Sambrook et al., 1989). After blotting was complete the membrane was washed for 15 min in 3X SSC with gentle shaking, air dried for 1 h, and baked at 80 °C for 2-3 h under vacuum.

After blotting to nitrocellulose and baking, the lane bearing the RNA size markers was cut from the blot and soaked in 5% acetic acid for 15 min. The nitrocellulose was then soaked in a solution of 0.04% methylene blue in 0.5 M sodium acetate pH 5.2 for 5-10 min, and destained with distilled H_2O. As the stained bands tend to fade with time, they were marked with pencil before storage.

Baked nitrocellulose membranes were prehybridized overnight at 42 °C in RNA hybridization medium adapted from Schloss *et al.* (1984) (Appendix 1). After prehybridization, the medium was changed and ^{32}P-labelled, nick translated probes, which had been denatured by heating in a boiling water bath for 10 min, were added. Hybridization was carried out for 48 h at 42 °C with gentle agitation. Hybridized membranes were washed twice for 15 min in 2X SSC, 0.1% SDS at room temperature, then twice for 20 min in 0.1X SSC, 0.1% SDS at 50 °C. Membranes were air-dried for 5 min, wrapped in Saran wrap, and exposed to Kodak X-Omat AR X-ray film with an intensifying screen at -70 °C.

Quantitation of tubulin mRNA by dot blot analysis

RNA samples were prepared as for RNA-formaldehyde gels with two exceptions: a) the samples were heated for 1 h at 50 °C, then chilled on ice, and b) the chilled samples were diluted 1:1 with 1X RNA buffer. The initial dot of each 1:1 serial dilution series contained 2.5 µg of RNA. RNA samples were spotted onto the B side of GeneScreen Plus membrane (Du Pont), which had been wetted by floating on distilled H_2O, and placed in a Bio-Rad Dot Blot manifold. The samples were left on the membrane for 30 min, after which gentle suction was applied. The membrane was air-dried for 1 h, then baked at 80 °C for 2-3 h. Prehybridization and hybridization were carried out as for Northern blots. Following autoradiography, the dots were cut out and counted in a liquid scintillation counter in toluene based scintillation fluid (Appendix 1). The autoradiographs were scanned with a Bio-Rad model 620 video densitometer using 1D-Analyst software.

***In vitro* translation of *Artemia* mRNA**

In vitro translation of poly(A)$^+$ RNA was carried out in a rabbit reticulocyte lysate system (Promega corp.). Five µg of RNA were translated in a 40 µL reaction mixture containing rabbit reticulocyte lysate, ~30 µCi (~0.75 mCi/mL) ^{35}S-methionine (Amersham) and other components as specified by the manufacturer. The translation mixture was incubated at 30 °C for 60 min; translation was stopped by chilling the mixture on ice.

For co-assembly with purified *Artemia* tubulin and subsequent electrophoresis on one-dimensional SDS-polyacrylamide gels, 20 µL of *in vitro* translation mixture were mixed with 1.8 mM GTP, 15 µg of purified *Artemia* tubulin, 10 µM taxol from 250 µM stock (Appendix 1) (the kind gift of Dr. M. Suffness, NCI, Bethesda, Md), and Pipes buffer pH 6.5 to give a total volume of 50 µL. Co-assembly reactions for two-dimensional gels were composed of 40 µL of *in vitro* translation mixture, 1.8 mM GTP, 10 µM taxol, 30 µg of purified *Artemia* tubulin, and at least 1 µL of 5X Pipes buffer pH 6.5 (Appendix 1) to give a total volume of 50-55 µL. The co-assembly mixtures were incubated at 37 °C for 40 min, then centrifuged through 400 µL 15-20% sucrose cushion pH 6.5 (Appendix 1) for 30 min at 39,000 x*g*, 20 °C. Pellets were rinsed gently with 150 µL of warm Pipes buffer containing 10 µM taxol, incubated in the rinse buffer for 10 min at 37 °C, and centrifuged at 39,000 x*g* for 10 min for 20 °C. For one-dimensional SDS-polyacrylamide gel electrophoresis, pellets were resuspended in 20 µL of Pipes buffer, pH 6.5, plus 20 µL of 2X sample buffer, on ice. Samples for two-dimensional gels were resuspended in 20 µL of Pipes buffer pH 6.5 and 20 µL of IEF overlay buffer, on ice.

Incorporation of ^{35}S-methionine into *in vitro* translated proteins was monitored using a method based on that provided by the supplier of the rabbit reticulocyte lysate translation system (Promega Corp.) One µL of the translation mixture was

added to 1.0 mL of 1 N NaOH, 1.5% H_2O_2 and incubated at 37 °C for 10 min, after which 4.0 mL of ice-cold 25% TCA containing 2% casamino acids were added. The solution was incubated on ice for 2.5 h. The precipitate was collected by suction on Whatman GF/C glass fiber filters and the filters rinsed with 10 mL of ice-cold 10% TCA followed by 5 mL of acetone. Filters were dried and counted in a toluene based scintillation fluid.

Fluorography

After electrophoresis, SDS-polyacrylamide gels containing ^{35}S- or ^{14}C-labelled proteins were stained, destained, and then soaked in Amplify (Amersham) for 30-40 min with gentle agitation. The gels were dried under vacuum at 80 °C for 5 h, then exposed to Kodak X-Omat AR X-ray film with an intensifying screen at -70 °C.

***In vivo* labelling of *Artemia* proteins**

Artemia were labelled *in vivo* with ^{14}C-sodium bicarbonate by placing 100 mg of hydrated *Artemia* cysts in 2 mL of hatch medium prepared as described in Appendix 1 but lacking sodium bicarbonate. Bicarbonate was supplied in the form of ^{14}C-bicarbonate (175 µCi, from a 2 mCi/mL stock, New England Nuclear). The *Artemia* were incubated as usual, filtered on 100 µm Nitex mesh, homogenized in 0.5 mL of Pipes buffer containing 4 M glycerol and 7 µL of each of the proteolytic enzyme inhibitor solutions A and B, and centrifuged as

described for the preparation of cell-free homogenates. Homogenates of dormant gastrulae were prepared by incubating 100 mg of hydrated cysts in ^{14}C-sodium bicarbonate-containing hatch medium for 5 min, then filtered and processed as usual. Protein concentrations were determined according to Lowry *et al.* (1951). The incorporation of ^{14}C into acid-precipitable material was determined by precipitating 5 µL of cell-free homogenate in 4 mL of ice-cold 25% TCA containing 2% casamino acids, followed by incubation on ice for 1 h. Precipitates were collected on Whatman GF/C glass fiber filters, washed with 10 mL ice-cold 10% TCA, then 5 mL acetone, dried, and counted in toluene-based scintillation cocktail.

Western blotting

Proteins were electrophoretically transferred from 10% SDS-polyacrylamide gels to nitrocellulose (Schleicher and Schull) according to the method of Towbin et al. (1979) in a Bio-Rad Trans-Blot cell. Transfer was carried out at 30 V with a 100 mA current limit in blotting electrode buffer (Appendix 1), for 16-20 h. Following blotting, nitrocellulose membranes were rinsed with TBS-Tween (Appendix 1). To ensure the proteins had been efficiently transferred the proteins were visualized using 0.2% Ponceau-S in 4% TCA. Proteins were resolved with distilled H_2O, and the membranes were completely destained in TBS-Tween at 4 °C. Occcasionally the polyacrylamide gels were stained with Coomassie Blue after blotting, as a further check that the

transfer of the proteins to the nitrocellulose was effective. If not used immediately, blots were stored at 4°C in TBS-Tween.

Horseradish peroxidase immunostaining

Proteins immobilized on nitrocellulose membranes were incubated with gentle agitation for 2-2.5 h in TBS-Tween with antibodies diluted as shown in Table 1. Following incubation with primary antibodies, blots were washed as follows, with gentle agitation: twice for 5 min in TBS-Tween, once for 5 min in HST (Appendix 1), and twice for 5 min in TBS-Tween. Species-specific horseradish peroxidase-conjugated secondary antibodies were diluted according to manufacturers' directions and incubated with Western blots in HST for 2 h with gentle agitation. Blots were washed as follows with gentle agitation: three times for 5 min in TBS-Tween, once for 10 min in HST, three times for 5 min in TBS-Tween, and once for 5 min in TBS (Appendix 1). Substrate solution was prepared (Appendix 1) and allowed to react with blots for up to 30 min. The reaction was stopped by placing the blots in distilled H_2O. Blots were photographed using Kodak Tri-X Pan Professional film.

Alkaline phosphatase immunostaining

Primary antibody incubation and washing were performed as for horseradish peroxidase immunostaining. Blots were incubated for 1.5 h with species-specific alkaline

Table 1. Anti-tubulin antibody dilutions, specificities, and sources.

ANTIBODY	SPECIES	DILUTION	SOURCE	SPECIFICITY	REFERENCE
polyclonal anti-tubulin	rabbit	1:100	Polysciences Inc.	α- and ß-tubulin	–
DM1A	mouse	1:500	Sigma Chemical Co.	α-tubulin	*J.Cell Biol.* **98**: 847-859 (1984)
TU-01	mouse	1:500-1:1000	V. Viklickey[a]	α-tubulin	*E.J.Cell Biol.* **41**: 82-88 (1986)
6-11B-1	mouse	1:15-1:20	G. Piperno[b]	acetylated α-tubulin	*J.Cell Biol.* **101**: 2085-2094 (1985)
YL1/2	rat	1:15-1:20	Sera-Lab	tyrosinated α-tubulin	*J.Cell Biol.* **93**: 576-582 (1982)
anti-Glu	rabbit	1:750-1:1000	J.C. Bulinski[c]	non-tyrosinated α-tubulin	*Cell* **38**: 779-789 (1984)
anti-Tyr	rabbit	1:1000	J.C. Bulinski	tyrosinated α-tubulin	*Cell* **38**: 779-789 (1984)
KMX	mouse	1:35-1:50	K. Gull[d]	ß-tubulin	*FEBS Lett.* **187**: 211-218 (1985)
DM1B	mouse	1:500	ICN Immuno-biologicals	ß-tubulin	*J.Cell Biol.* **95**: 229a (1982)
TUB2.1	mouse	1:200	Sigma Chemcial Co.	ß-tubulin	*Proc.Natl.Acad.Sci.*(USA) **79**: 2579-2583 (1986)

[a] Institute of Molecular Genetics, Czechoslovak Academy of Sciences, CS-14220
[b] The Rockefeller University, New York, NY
[c] Dept. of Anatomy and Cell Biology, Columbia University, College of Physicians and Surgeons, New York, NY
[d] Dept. of Biochemistry and Molecular Biology, School of Biological Sciences, University of Manchester, Manchester, England

phosphatase conjugated secondary antibodies (Promega, Sigma Chemical Company) diluted according to manufacturer's directions in TBS-Tween, with the exception of those blots which had been incubated with TU-01, anti-Glu, or anti-Tyr primary antibodies. In these cases, the anti-rabbit secondary antibodies were incubated in HST. Blots were rinsed with gentle agitation 3 times for 5 min in TBS-Tween, once for 5 min in TBS, then reacted with freshly prepared substrate solution (Appendix 1). The reaction was stopped by placing the blots in alkaline phosphatase stop buffer. Blots were photographed using Kodak Tri-X Pan professional film.

Carboxypeptidase A treatment of *Artemia* tubulin on western blots

Western blots were treated with carboxypeptidase A based on a procedure described by Gundersen *et al.* (1987). Blots were blocked by incubation in 10% normal rabbit serum for 30 min with gentle shaking. Following a brief rinse in 25 mM TBS (Appendix 1), blots were incubated in 25 mM TBS containing 10 µg/mL (0.6 units/mL) PMSF-treated carboxypeptidase A type II (Sigma, for 30 min at 37 °C with occasional agitation. Blots were briefly rinsed in 25 mM TBS, re-blocked, rinsed again with 25 mM TBS, then with TBS-Tween, and immunostained. Control blots were processed in the same way, but were incubated in 25 mM TBS minus carboxypeptidase A.

RESULTS

RNA purification

RNA was isolated from dormant gastrulae and from organisms developed 15 h and 24 h. Oligo-dT cellulose chromatography was used to isolate poly(A)$^+$-enriched RNA, henceforth referred to as poly(A)$^+$ RNA. The total cytoplasmic RNA fraction contained all RNA present in the cytoplasm, including that present in polysomes. Polysomal RNA was also isolated. Consistent yields of RNA were obtained for both total cytoplasmic RNA and polysomal RNA fractions. Tables 2 and 3 show yields for representative cytoplasmic and polysomal RNA preparations respectively. The A_{260}/A_{280} ratios of the poly(A)$^+$ preparations were consistent with pure RNA, falling on or very near 1.8. The yield of poly(A)$^+$ RNA from 24 h organisms was considerably lower than that from 15 h organisms, for both total cytoplasmic and polysomal RNA preparations. Also, the yield of polysomal poly(A)$^+$ RNA was lower for 0 h organisms than for 15 h organisms.

Northern blot analysis of *Artemia* tubulin mRNA in total cytoplasmic RNA preparations

Equal amounts (5 µg) of poly(A)$^+$ or poly(A)$^-$ RNA were applied to individual lanes of 1.5% agarose gels containing 6% formaldehyde. Following electrophoresis, transfer to nitrocellulose, and hybridization with ^{32}P-labelled *Drosophila* tubulin gene clones contained in plasmids pDmTα1 and DTB2,

Table 2. Yield of total cytoplasmic RNA per gram *Artemia* (wet weight after incubation)

Development Time	Total RNA (mg)	Poly(A)$^+$ RNA (µg)
0 h	2.9	46.7
15 h	2.4	48.8
24 h	1.5	31.2

RNA was prepared as described in Materials and Methods. Data represent the yield from a typical total cytoplasmic RNA preparation. Quantitation of RNA was based on absorbance of RNA solutions at 260 nm as described in Sambrook *et al.* (1989).

Table 3. Yield of polysomal RNA per gram *Artemia* (wet weight after incubation)

Development time	Total RNA (mg)	Poly(A)$^+$ RNA (µg)	% of total cytoplasmic mRNA
0 h	1.1	10.7	23
15 h	1.3	23.9	49
24 h	1.0	12.5	40

RNA was prepared as described in Materials and Methods. Data represent the yield from a typical polysomal RNA preparation. Quantitation of RNA was based on absorbance of RNA solutions at 260 nm as described in Sambrook *et al.* (1989).

Figure 1. Northern blot analysis of *Artemia* total cytoplasmic poly(A)$^+$ RNA. Total cytoplasmic poly(A)$^+$ RNA from organisms developed 0 h (lane 1), 15 h (lane 2), and 24 h (lane 3) was electrophoresed on 1.5% denaturing agarose gels, transferred to nitrocellulose, and hybridized with ^{32}P-labelled plasmids pDmTα1 (α) and DTB2 (β). Five μg of RNA were loaded per lane. A single band of approximately 1.9 kB was seen for both *Artemia* α- and β-tubulin mRNA. Size markers in kB are shown on the left side of the autoradiogram.

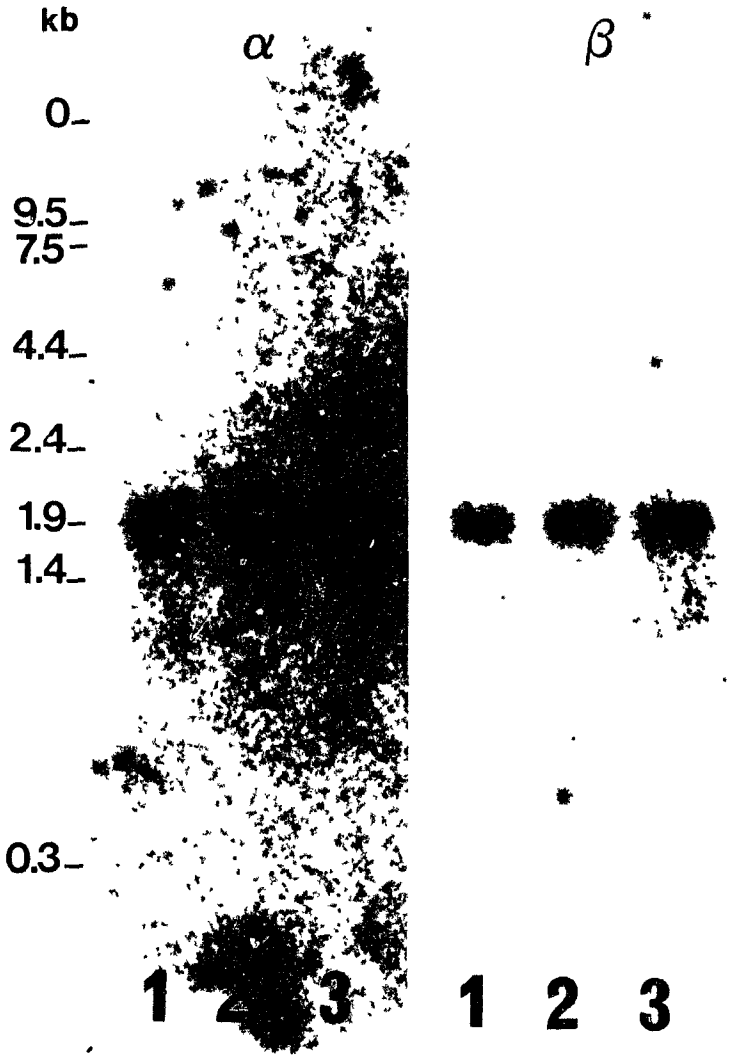


Figure 1.

one size class of tubulin mRNA approximately 1.9 kB in size was revealed on autoradiograms (Fig.1). The signal intensity of the α- and β-tubulin mRNA bands, as well as the size, were similar for poly(A)$^+$ RNA from organisms developed 0, 15 or 24 h. Total cytoplasmic poly(A)$^-$ RNA hybridized much less strongly to the ^{32}P-labelled plasmids (Fig.2) indicating that the poly(A)$^-$ RNA fraction contained little tubulin mRNA. The very faint single bands visible on the poly(A)$^-$ RNA autoradiograms, however, were in the same position as the tubulin mRNA bands seen on the poly(A)$^+$ RNA autoradiograms.

^{32}P-labelled, nick-translated plasmids pDmTα1 and DTB2 hybridized equally well to the 1.9 kB α- and β-tubulin mRNA bands on Northern blots as did similarly labelled *Drosophila* α- and β-tubulin gene clone fragments composed only of coding sequence (not shown). Once again, the intensity of the signal was the same for RNA from organisms developed 0, 15, and 24 h. Whole plasmids were therefore used for all hybridizations.

Quantitation of tubulin mRNA

Dot blots of serially diluted total cytoplasmic poly(A)$^+$ RNA were hybridized with ^{32}P-labelled, nick-translated *Drosophila* tubulin gene clones contained in plasmids pDmTα1 and DTB2 (Fig.3). Approximately the same signal intensity was seen for tubulin mRNA in organisms developed 0, 15 and 24 h. Liquid scintillation spectrophotometry of the hybridized dots (Fig.4), and analysis of the autoradiograms using a

Figure 2. Northern blot analysis of *Artemia* total cytoplasmic poly(A)$^-$ RNA. Total cytoplasmic poly(A)$^-$ RNA from organisms developed 0 h (lane 1), 15 h (lane 2), and 24 h (lane 3) was electrophoresed on 1.5% denaturing agarose gels, transferred to nitrocellulose, and hybridized with ^{32}P-labelled plasmids pDmTα1 (α) and DTB2 (β). Five μg of RNA were loaded per lane. Very faint α- and β-tubulin mRNA bands approximately 1.9 kB in size were seen. Figure 1 and Figure 2 were hybridized together and exposed for the same length of time. Size markers in kB are shown on the left side of the autoradiogram.

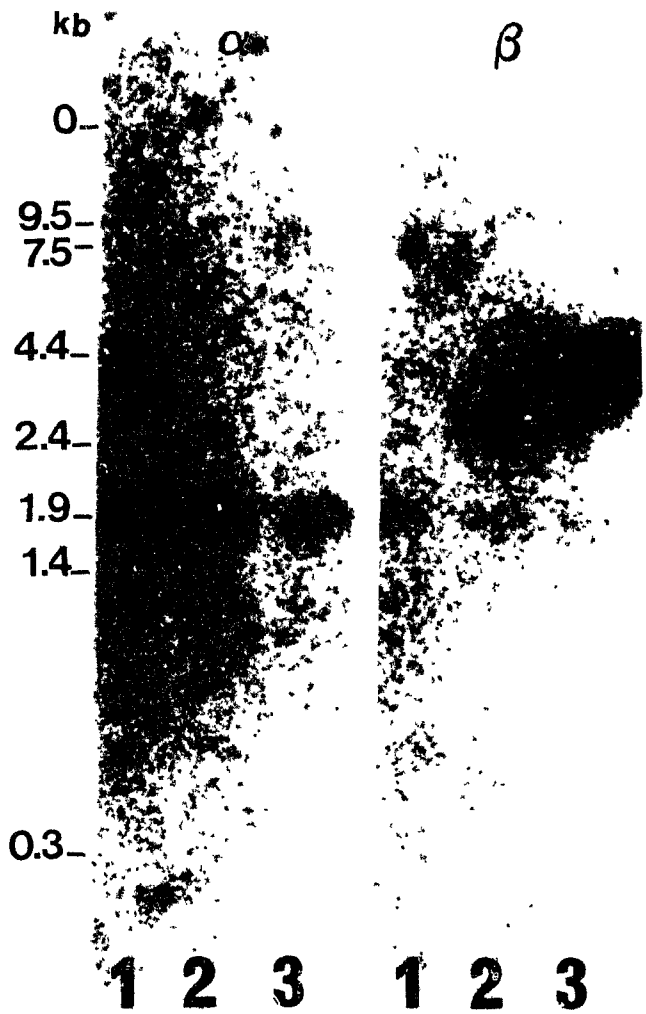


Figure 2.

Figure 3. Dot-blot analysis of *Artemia* tubulin mRNA. Total cytoplasmic poly(A)$^+$ RNA from organisms developed 0, 15, and 24 h (as labelled) was serially diluted, dotted onto Gene-Screen Plus, and hybridized to ^{32}P-labelled cloned *Drosophila* α- and β-tubulin genes contained in plasmids pDmTα1 (α) and DTB2 (β) respectively. The first, undiluted dot contained 2.5 μg of RNA.

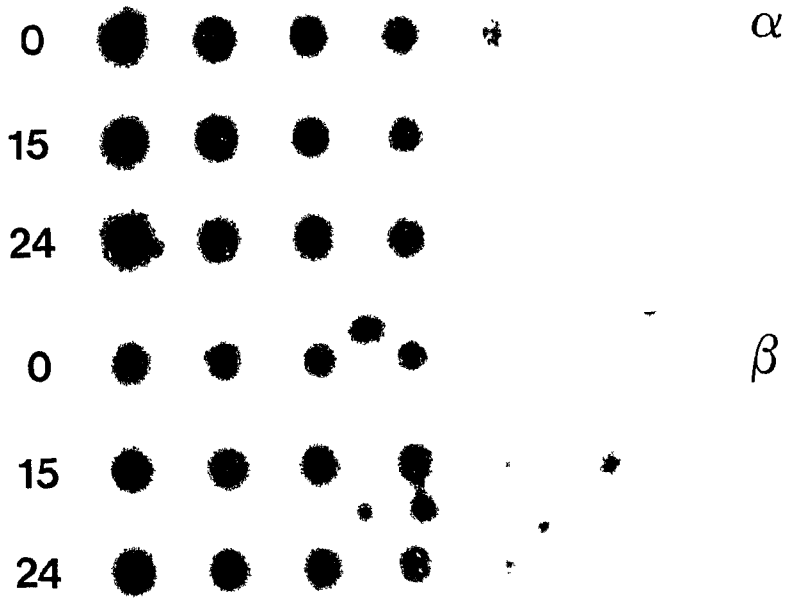


Figure 3.

Figure 4. The amount of *Artemia* tubulin mRNA is constant during early post-gastrular development. Total cytoplasmic poly(A)$^+$ RNA from organisms developed 0, 15, and 24 h (as labelled) was serially diluted, applied to GeneScreen Plus, hybridized to ^{32}P-labelled plasmid pDmTα1 (A) and DTB2 (B), and the hybridized spots cut out and subjected to liquid scintillation spectrophotometry.

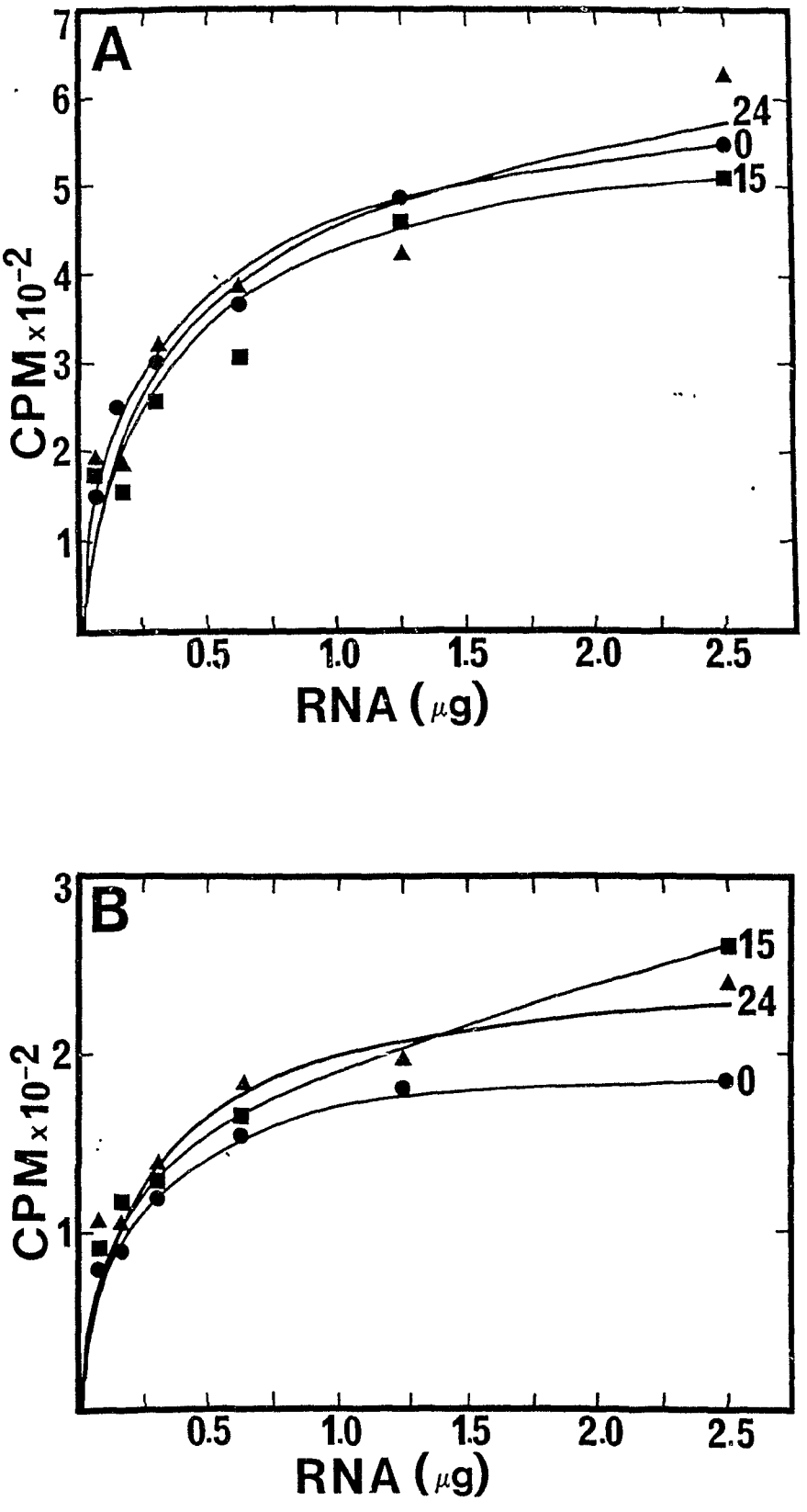


Figure 4.

Figure 5. Densitometric analysis of the relative amount of α-tubulin mRNA during *Artemia* post-gastrular development. Total cytoplasmic poly(A)$^+$ RNA from organisms developed 0, 15, and 24 h (as labelled) was serially diluted, applied to GeneScreen Plus, hybridized with the ^{32}P-labelled plasmid pDmTα1, and the resultant autoradiogram scanned with a Bio-Rad model 620 video densitometer. The dots were also cut out and analysed by liquid scintillation spectrophotometry, which yielded the same results.

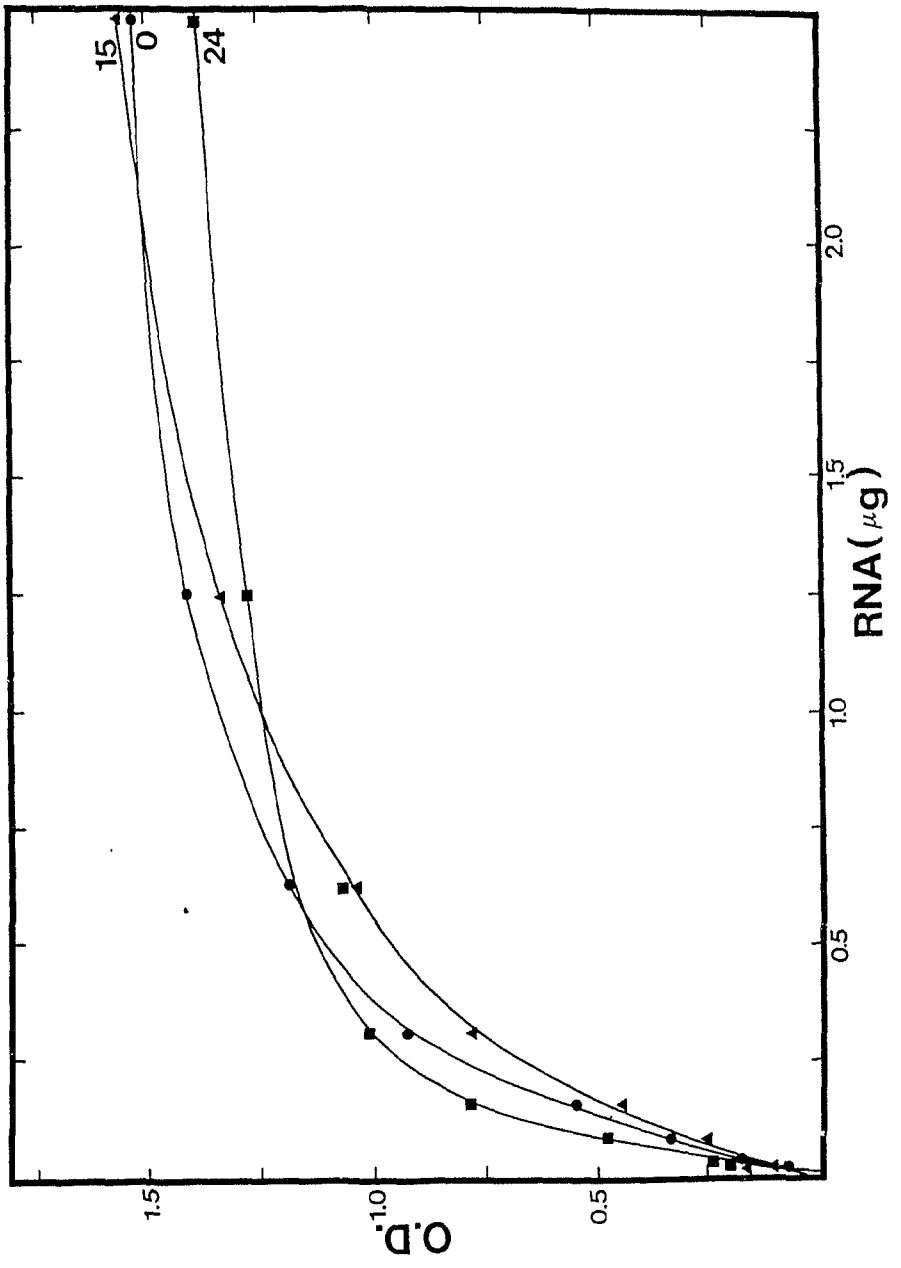


Figure 5.

Figure 6. Densitometric analysis of the relative amount of β-tubulin mRNA during *Artemia* post-gastrular development. Total cytoplasmic poly(A)$^+$ RNA from organisms developed 0, 15, and 24 h (as labelled) was serially diluted, applied to GeneScreen Plus, hybridized with the ^{32}P-labelled plasmid DTB2, and the resultant autoradiogram scanned with a Bio-Rad model 620 video densitometer. The dots were also cut out and analysed by liquid scintillation spectrophotometry, which yielded the same results.

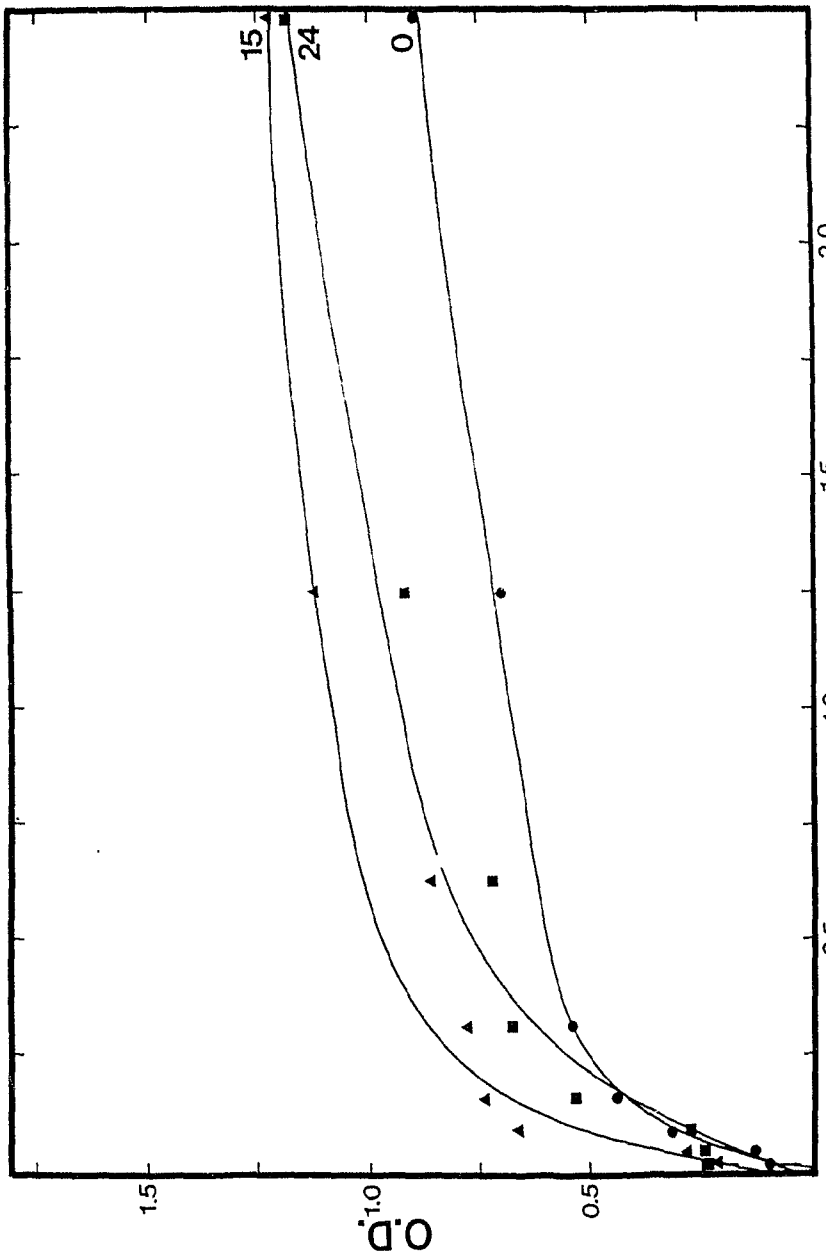


Figure 6.

Bio-Rad model 620 video densitometer with 1D-Analyst software (Figs.5 and 6) both revealed, with one exception, no appreciable difference in the amount of α- or β-tubulin mRNA in poly(A)$^+$ RNA prepared from total cytoplasmic RNA. β-tubulin mRNA from dormant gastrulae (0 h) appeared to be somewhat less abundant than β-tubulin mRNA from 15 h and 24 h of development. However, this result may be slightly aberrant, as 0 h β-tubulin mRNA frequently appeared to be equally abundant as 15 h and 24 h β-tubulin mRNA on other blots.

In vitro translation of *Artemia* poly(A)$^+$ mRNA prepared from total cytoplasmic RNA

Artemia poly(A)$^+$ mRNA was isolated from total cytoplasmic RNA by oligo-dT cellulose chromatography and translated in a rabbit reticulocyte lysate system. The amount of poly(A)$^+$ RNA required to achieve optimal incorporation of ^{35}S-methionine into acid-precipitable material was determined (Fig.7). 5 μg of poly(A)$^+$ RNA were used for subsequent translations.

A comparison of the translation products of poly(A)$^+$ and poly(A)$^-$ RNA from organisms developed for 15 h, and controls lacking RNA is shown in Figure 8. One very faint band may be seen in lane 1, indicating a very low level of endogenous protein synthesis in the rabbit reticulocyte lysate translation system. By comparing lanes 2 and 3 it is clear that the poly(A)$^+$ RNA fraction (lane 2) contained much more

Figure 7. Optimization of translation of *Artemia* poly(A)$^+$ RNA. Increasing amounts of poly(A)$^+$ RNA from organisms developed 15 h were translated *in vitro* in rabbit reticulocyte lysate. The incorporation of ^{35}S-methionine into acid-precipitable material in 1 µL of translation mixture was determined (see Materials and Methods) at the beginning of incubation (●——●) and after 60 min at 30 °C (▲——▲).

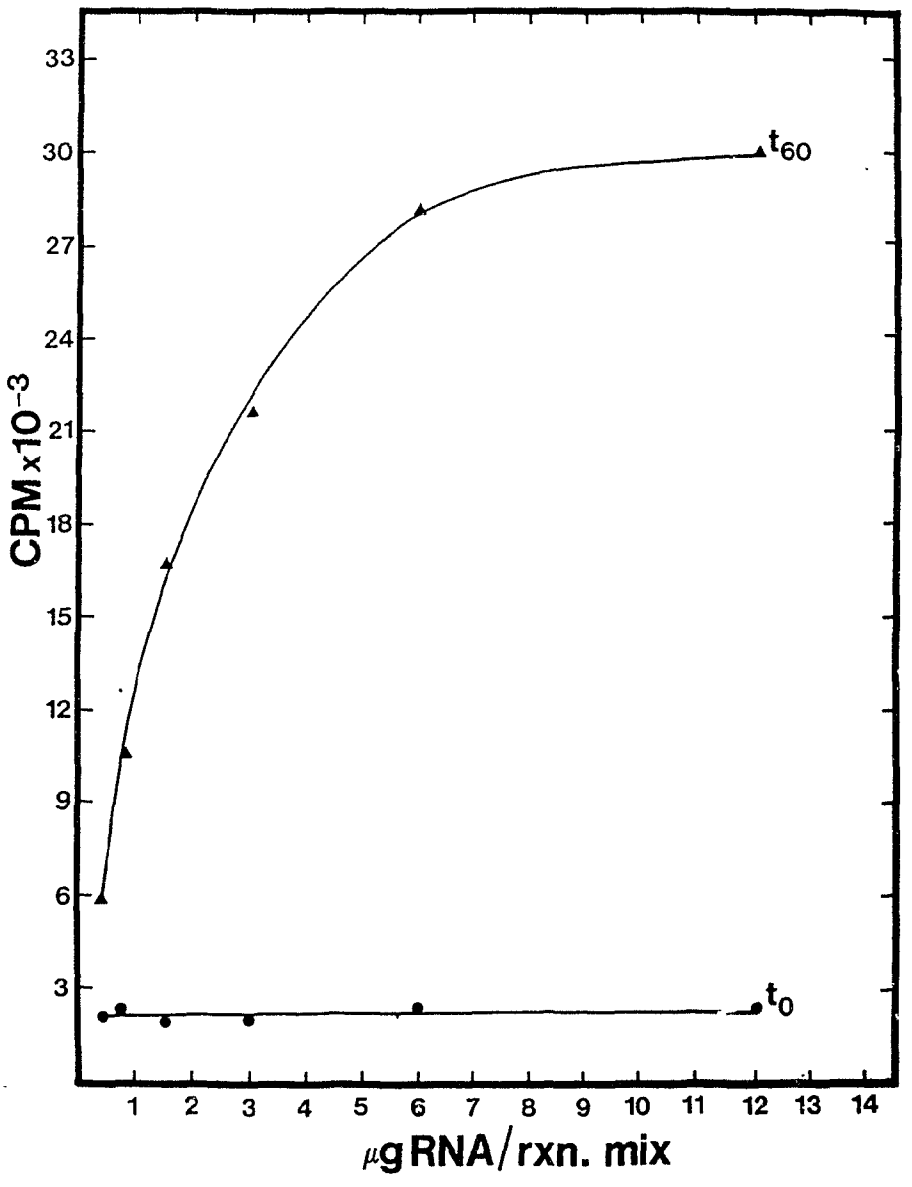

33
30
27
24
21
18
15
12
9
6
3
CPM x10⁻³
t60
t0
1 2 3 4 5 6 7 8 9 10 11 12 13 14
μg RNA/rxn. mix

Figure 7.

Figure 8. *Artemia* poly(A)$^+$ RNA translated more effectively than poly(A)$^-$ RNA in rabbit reticulocyte lysate. Five µg of total cytoplasmic poly(A)$^+$ RNA (lane 2) and poly(A)$^-$ RNA (lane 3) were translated *in vitro* in rabbit reticulocyte lysate. No RNA was added to the translation reaction in lane 1. The ^{35}S-labelled translation products were electrophoresed on 10% SDS-polyacrylamide gels. The figure is a fluorogram of the translation products after electrophoresis. The molecular masses of standards (kilodaltons) are shown on the left side of the figure.

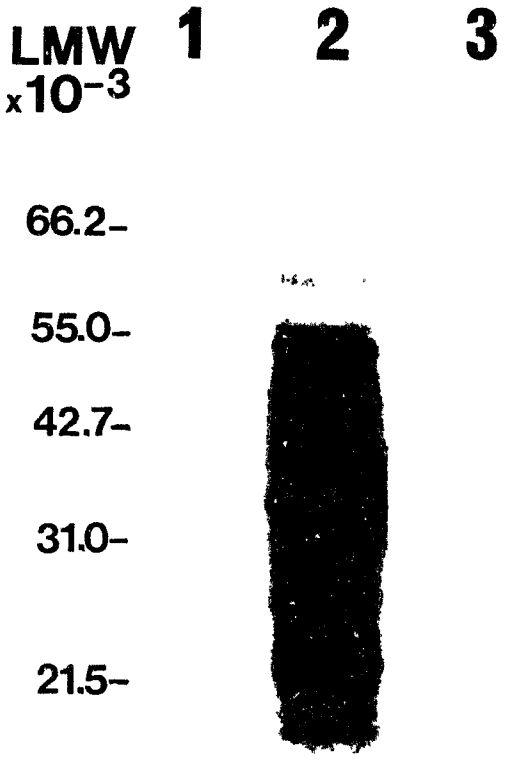


Figure 8.

Figure 9. *In vitro* translation of *Artemia* total cytoplasmic RNA and co-assembly of the translation products with *Artemia* tubulin. Total cytoplasmic poly(A)$^+$ RNA from organisms developed 0 h (lane 1), 15 h (lane 2), and 24 h (lane 3) was translated *in vitro* in rabbit reticulocyte lysate. The products were either separated on a 10% SDS-polyacrylamide gel (panels A and B), or co-assembled with unlabelled 15 h *Artemia* tubulin (panels C and D) prior to electrophoresis, as described in Materials and Methods. Panels A and C show the Coomassie blue stained gel; panels B and D show the corresponding fluorograms. The molecular masses of standards (kilodaltons) are shown on the left side of the figure. The positions of the α- and β-tubulins are indicated. TUB, tubulin.

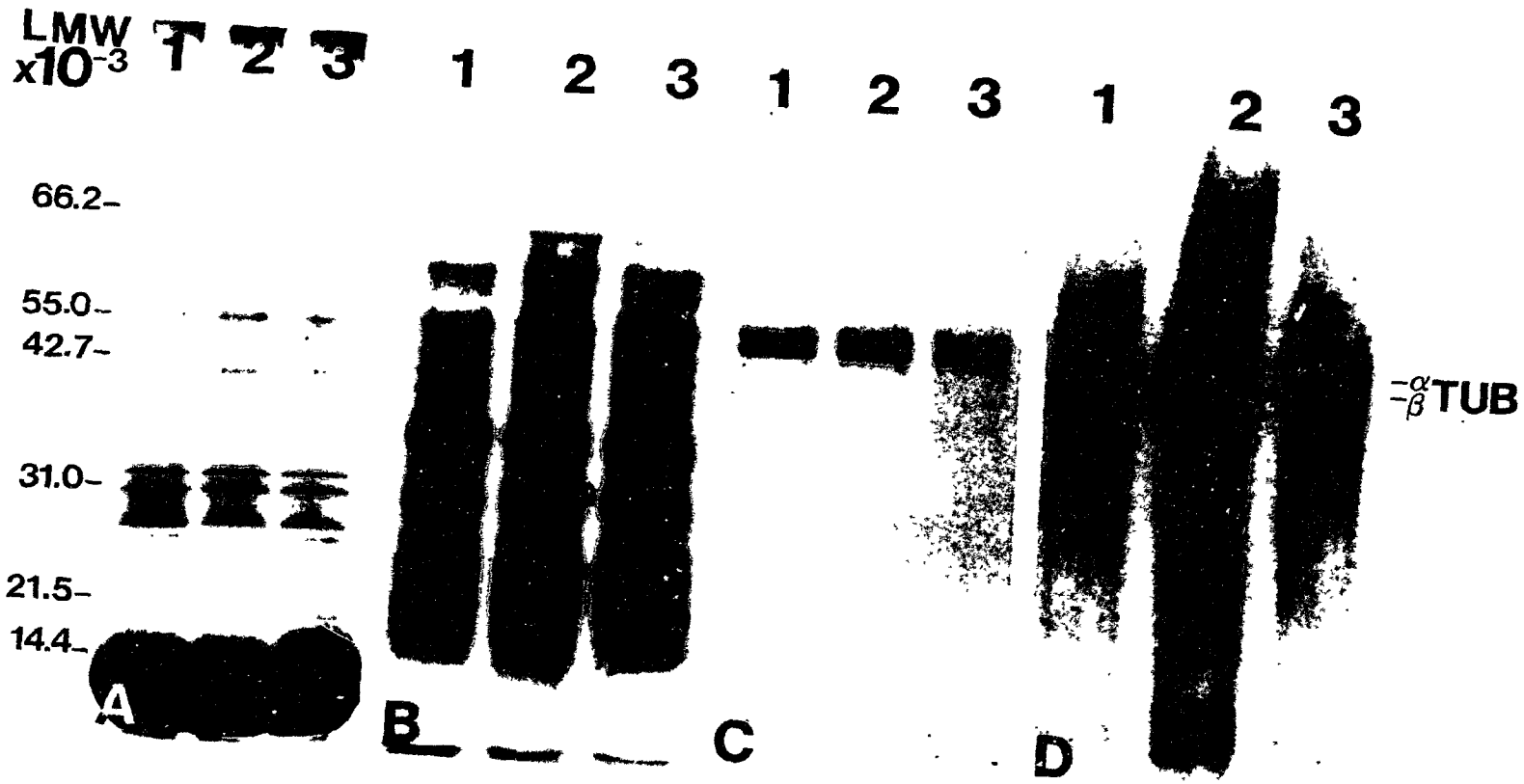


Figure 9.

translatable mRNA than did the poly(A)$^-$ RNA fraction (lane 3). All lanes were exposed for an equal length of time.

In vitro translation of poly(A)$^+$ RNA yielded a large number of ^{35}S-methionine-labelled polypeptides over a range of molecular weights, from approximately 17 kD to over 80 kD (Fig.9, panel B). *In vitro* translation of equivalent amounts of poly(A)$^+$ RNA from organisms developed for 15 h and 24 h typically yielded more acid-precipitable material than did 0 h poly(A)$^+$ RNA (Table 4). As the tubulin synthesized *in vitro* could not be resolved, on either one- or two-dimensional gels (Fig.9, panels A and B, and Fig.10), it was purifi d by taxol-induced co-polymerization with non-rabiolabelled *Artemia* tubulin followed by centrifugation through 15-20% sucrose cushions (Fig. 9, panels C and D). When tubulin synthesized by *in vitro* translation of poly(A)$^+$ RNA from organisms developed 0, 15, and 24 h was co-assembled with purified 0 h, 15 h, and 24 h tubulin respectively, the same results were obtained as when only 15 h purified tubulin was used. All co-assemblies were therefore carried out with 15 h tubulin, as it was more efficiently purified than tubulin from organisms developed either 0 h or 24 h.

A Coomassie blue stained one-dimensional gel of co-assembled tubulins in Figure 9, panel C shows α- and β-tubulins clearly separated from other proteins. The corresponding fluorogram (Fig.9, panel D) revealed one labelled α-tubulin band and one labelled β-tubulin band synthesized from

Table 4. Incorporation of ^{35}S-methionine into acid-precipitable material during *in vitro* translation of *Artemia* total cytoplasmic poly(A)$^+$ RNA

RNA	cpm t_o	cpm t_{60}
0 h	1.9×10^3	3.8×10^4
15 h	1.9×10^3	6.2×10^4
24 h	2.8×10^3	6.9×10^4

Data represent the cpm in 1 µL of typical 40 µL *in vitro* translation reactions, determined as described in Materials and Methods.

Figure 10. Two-dimensional gel electrophoresis of *Artemia in vitro* translation products. Five µg of total cytoplasmic poly(A)$^+$ RNA from organisms developed 15 h were translated *in vitro* in rabbit reticulocyte lysate. The translation products were analysed in one direction by isoelectric focusing (pH), and in the other direction by SDS-polyacrylamide gel electrophoresis (SDS). The pH decreased left to right, from approximately 6.5 to approximately 5. The figure is the fluorogram of the two-dimensional gel. The boxed-in area indicates the region in which the *in vitro* translated tubulins are likely to be located.

Figure 10.

poly(A)$^+$ RNA from organisms developed 0, 15, and 24 h. A small amount of degraded labelled tubulin or prematurely terminated tubulin polypeptides appear to have purified with the intact tubulin, as evidenced by the trailing seen below the β-tubulin band.

Coomassie blue stained gels of *in vitro* translated tubulins co-assembled with purified tubulin showed the same pattern of α- and β-tubulin spots on two-dimensional gels as did purified tubulin alone but, as a smaller amount of tubulin is present, were much more lightly stained (compare Fig.11, panel A, with Fig.24, panel A). A single α-tubulin spot and a maximum of two β-tubulin spots were seen on the corre-sponding fluorograms of the two-dimensional Coomassie blue stained gels (Fig.11, panel B). When the exposure time of the fluorogram was reduced, only a single α-tubulin and a single β-tubulin were resolved in the *in vitro* translation products of poly(A)$^+$ RNA. *Artemia*, then, synthesize one α-tubulin and one or two β-tubulins from each of the single size classes of α- and β-tubulin mRNA, as resolved on the two-dimensional gel system used.

Northern blot analysis of *Artemia* polysomal RNA

In order to determine whether or not tubulin mRNA is translated during the first 24 h of *Artemia* post-gastrular development, polysomal poly(A)$^+$ RNA was isolated. Polysomal and total cytoplasmic poly(A)$^+$ RNAs from organisms developed 0, 15, and 24 h of development were analysed in parallel on

Figure 11. Two-dimensional analysis of *Artemia* tubulin translated *in vitro*. Five µg of total cytoplasmic poly(A)+ mRNA from organisms developed 0 h, 15 h, and 24 h (as labelled) were translated *in vitro* in rabbit reticulocyte lysate, co-assembled with unlabelled 15 h *Artemia* tubulin, then analysed in one direction by isoelectric focusing (pH) and in the other direction by SDS-polyacrylamide gel electrophoresis (SDS). The pH decreased left to right, from approximately 5.6 to approximately 5.1. Panel A shows the Coomassie Blue stained gels; panel B shows the corresponding fluorograms Shorter exposures failed to resolve the labelled tubulins into more than one spot. Only the tubulin region of the gels is shown as there were no other labelled polypeptides visible on the fluorogram. The arrows in panel B point to an unidentified translation product.

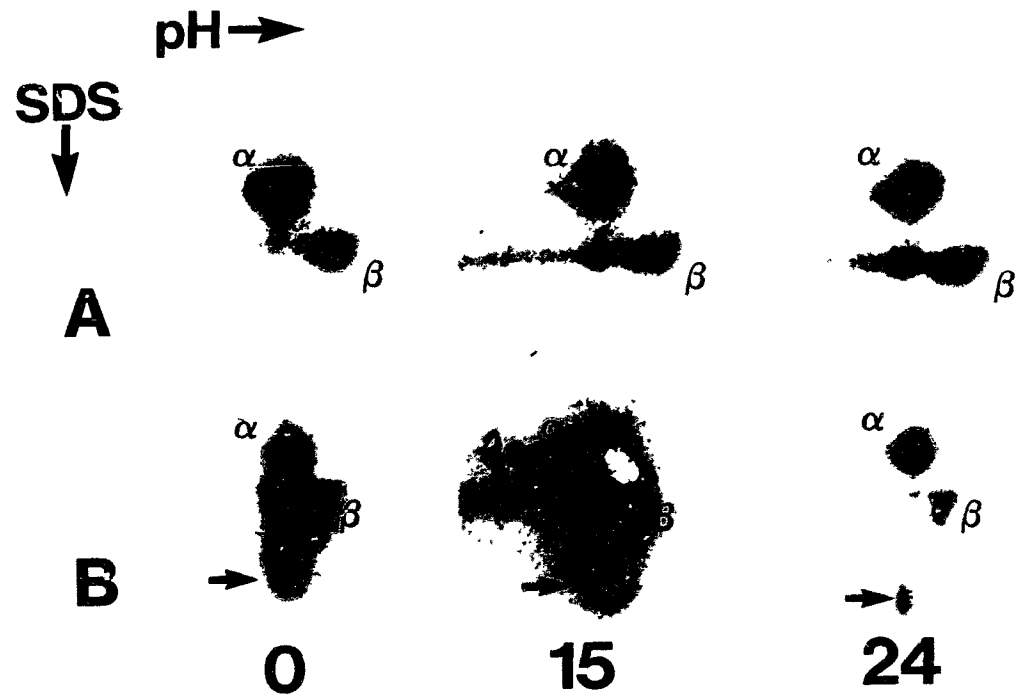


Figure 11.

Figure 12. Comparison of *Artemia* α-tubulin mRNA in total cytoplasmic and polysomal RNAs. Total cytoplasmic (lanes 1, 3, 5) and polysomal (lanes 2, 4, 6) poly(A)$^+$ RNAs from organisms developed 0 h (lanes 1, 2), 15 h (lanes 3, 4), and 24 h (lanes 5, 6) were electrophoresed on 1.5% denaturing agarose gels, transferred to nitrocellulose, and hybridized with the ^{32}P-labelled plasmid pDmTα1. Five μg of RNA were loaded per lane. A single α-tubulin mRNA approximately 1.9 kB in length was seen in the total cytoplasmic RNA, but was only barely detectable in the polysomal RNA. Size markers in kB are shown on the left side of the autoradiogram.

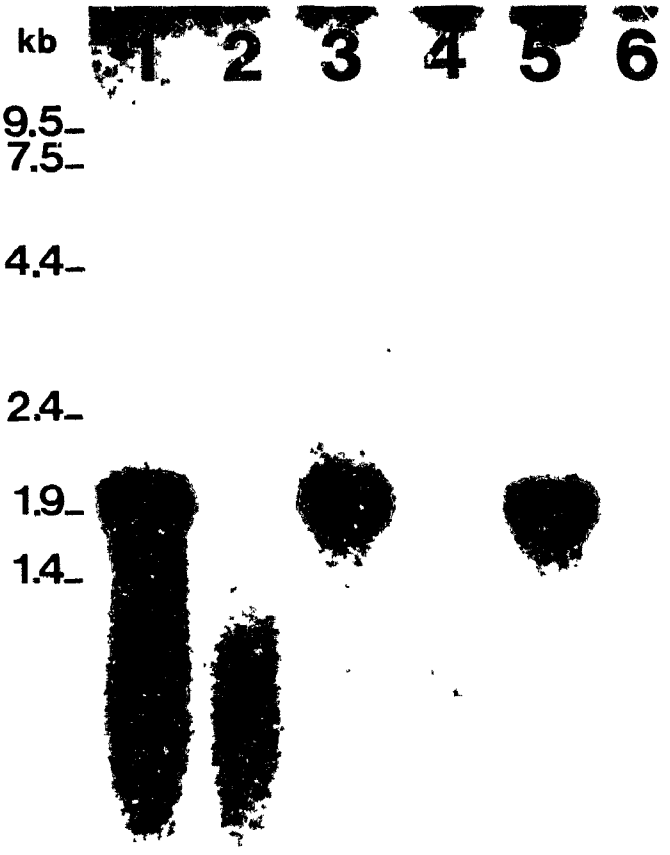


Figure 12.

Northern blots. The autoradiogram showed that the polysomal poly(A)$^+$ RNA contained only a very small amount of *Artemia* α-tubulin mRNA capable of hybridizing to the cloned *Drosophila* α-tubulin gene, when compared to the very intense signal seen in the total cytoplasmic RNA lanes (Fig.12). In fact, it initially appeared that there was no α-tubulin mRNA in the polysomal poly(A)$^+$ RNA. It was necessary to overexpose the Northern blot with respect to the total cytoplasmic RNA lanes, in order that the very light bands in the polysomal RNA lanes could be readily detected (Fig.13). The α-tubulin mRNA bands were in identical positions in both the total cytoplasmic and polysomal RNA lanes, at approximately 1.9 kB. The lengthy exposure of the blot in Figure 13 revealed that there may be some degradation of the poly(A)$^+$ RNAs, as seen by the trailing below the 1.9 kB bands.

Northern blots identical to those used for characterization of α-tubulin mRNA were probed with the ^{32}P-labelled cloned *Drosophila* β-tubulin gene contained in plasmid DTB2. The autoradiogram, in this case, did not reveal the presence of the *Artemia* β-tubulin mRNA in the polysomal RNA lanes (Fig.14) indicating, as for α-tubulin, that a low level of β-tubulin mRNA - or none at all - was being translated. In order to confirm the synthesis of α-tubulin in developing *Artemia* and to test whether β-tubulin is also synthesized, *in vitro* translation of polysomal poly(A)$^+$ RNA and *in vivo* labelling of *Artemia* proteins were carried out.

Figure 13. *Artemia* tubulin mRNA is more abundant in total cytoplasmic poly(A)$^+$ RNA than in polysomal poly(A)$^+$ RNA. An identical autoradiogram as that seen in Fig.12 was overexposed with respect to the total cytoplasmic RNA lanes (lanes 1, 3, 5) in order to reveal a very faint α-tubulin mRNA band of the same size as that in the total cytoplasmic lanes (1.9 kB) in the polysomal RNA lanes (lanes 2, 4, 6).

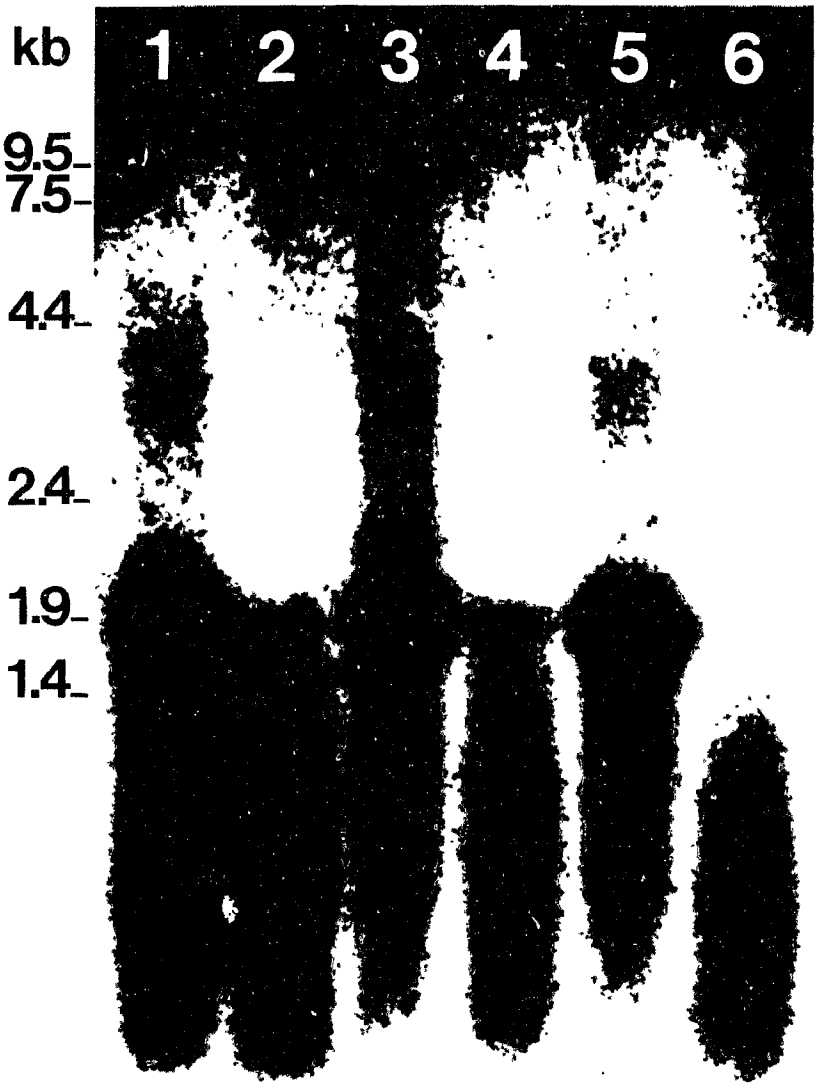


Figure 13.

Figure 14. Comparison of *Artemia* β-tubulin mRNA in total cytoplasmic and polysomal RNAs. Total cytoplasmic (lanes 1, 3, 5) and polysomal (lanes 2, 4, 6) poly(A)$^+$ RNAs from organisms developed 0 h (lanes 1, 2), 15 h (lanes 3, 4), and 24 h (lanes 5, 6) were electrophoresed on 1.5% denaturing agarose gels, transferred to nitrocellulose, and hybridized with the ^{32}P-labelled plasmid DTB2. Five μg of RNA were loaded per lane. A single β-tubulin mRNA approximately 1.9 kB in length was seen in the total cytoplasmic RNA, but not in the polysomal RNA. Size markers in kB are shown on the left side of the autoradiogram.

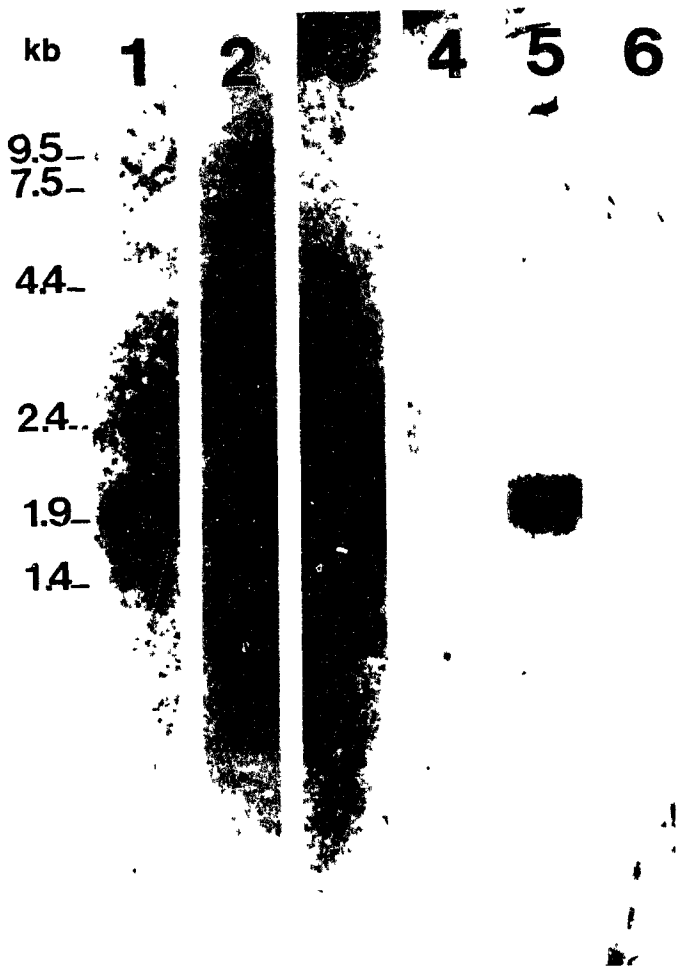


Figure 14.

Table 5. Incorporation of ^{35}S-methionine into acid-precipitable material during *in vitro* translation of *Artemia* polysomal poly(A)$^+$ RNA.

RNA	cpm t_o	cpm t_{60}
0 h	4.6×10^3	2.4×10^4
15 h	2.6×10^3	7.7×10^4
24 h	3.3×10^3	5.3×10^4

Data represent the cpm in 1 µL of typical 40 µL *in vitro* translation reactions, determined as described in Materials and Methods.

***In vitro* translation of *Artemia* polysomal RNA**

Polysomal poly(A)$^+$ RNA was translated *in vitro* following the same procedure as for total cytoplasmic RNA. The incorporation of ^{35}S-methionine into acid-precipitable material in a typical *in vitro* translation experiment is shown in Table 5. As was the case with total cytoplasmic poly(A)$^+$ RNA, *in vitro* translation of polysomal poly(A)$^+$ RNA from dormant gastrulae (0 h) yielded less acid-precipitable counts than did 15 h and 24 h polysomal poly(A)$^+$ RNA.

A wide range of polypeptides was translated *in vitro* from polysomal poly(A)$^+$ RNA, as seen in Figure 15. The translation products were therefore co-assembled with unlabelled *Artemia* tubulin, to allow the analysis of polysomal RNA-encoded tubulin. As shown in Figure 16, *in vitro* synthesized tubulin was present in only a small amount. A comparison was made of the relative amounts of tubulin synthesized *in vitro* from the same amount (5 µg) of 15 h total cytoplasmic, and 0, 15 and 24 h polysomal poly(A)$^+$ RNAs. The result of co-assembly of each of the translation products with unlabelled *Artemia* tubulin is seen in Figure 17. Assuming equal efficiency of co-assembly in the taxol-driven assembly reactions, a much larger amount of tubulin was synthesized from the total cytoplasmic RNA. For reasons which are not clear, the sucrose cushion in this case did not prevent the sedimentation of a number of lab led, non-tubulin proteins along with the products of the assembly

Figure 15. *In vitro* translation of *Artemia* polysomal RNA. Five μg of polysomal poly(A)$^+$ RNA from organisms developed 0 h (lane 1), 15 h (lane 2), and 24 h (lane 3) were translated *in vitro* in rabbit reticulocyte lysate. Translation products were electrophoresed on a 10% SDS-polyacrylamide gel. Panel A shows the Coomassie Blue stained gel; panels B shows the corresponding fluorogram. The molecular masses of standards (kilodaltons) are shown on the left side of the figure.

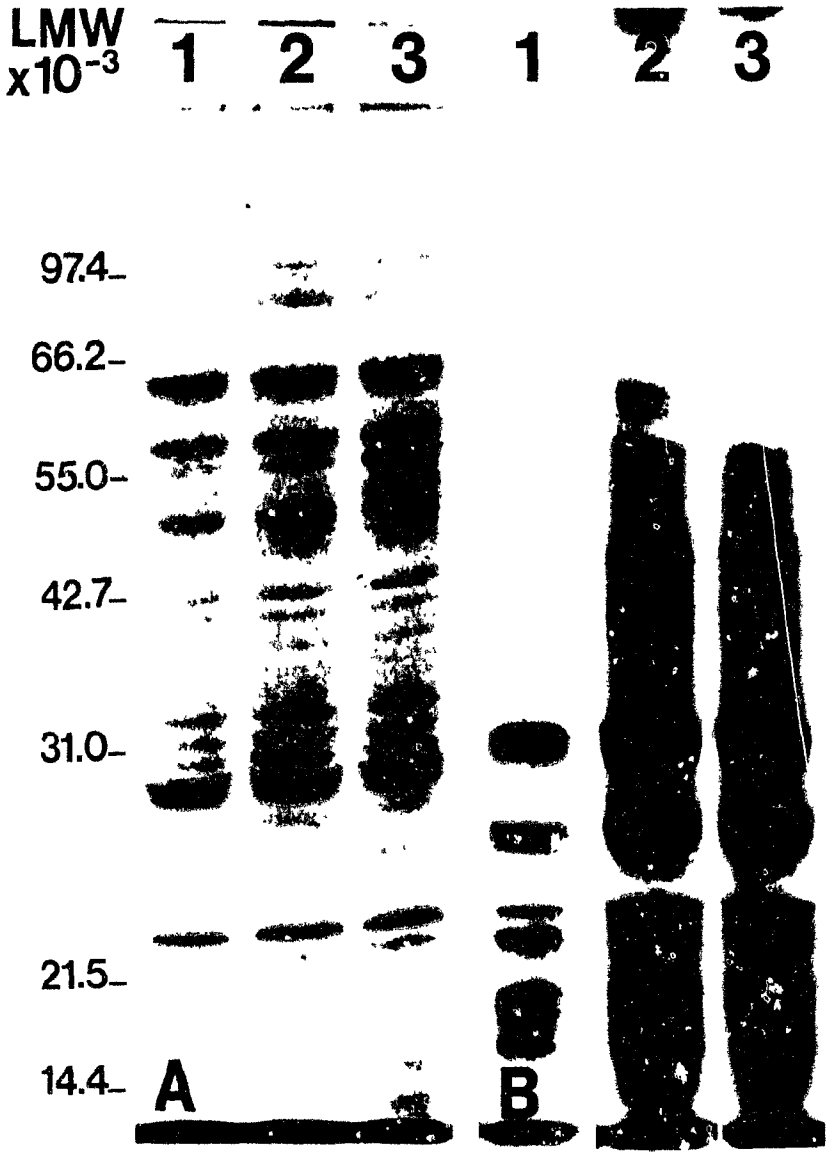


Figure 15.

Figure 16. *In vitro* translation of *Artemia* polysomal RNA and co-assembly of the translation products with *Artemia* tubulin. *Artemia* polysomal poly(A)$^+$ RNA from organisms developed 0 h (lane 1), 15 h (lane 2), and 24 h (lane 3) was translated *in vitro* in rabbit reticulocyte lysate, then co-assembled with unlabelled 15 h *Artemia* tubulin and electrophoresed on a 10% SDS-polyacrylamide gel. Panel A shows the Coomassie Blue stained gel; panel B shows the corresponding fluorogram. The molecular masses of standards (kilodaltons) are shown on the left side of the figure. TUB, tubulin.

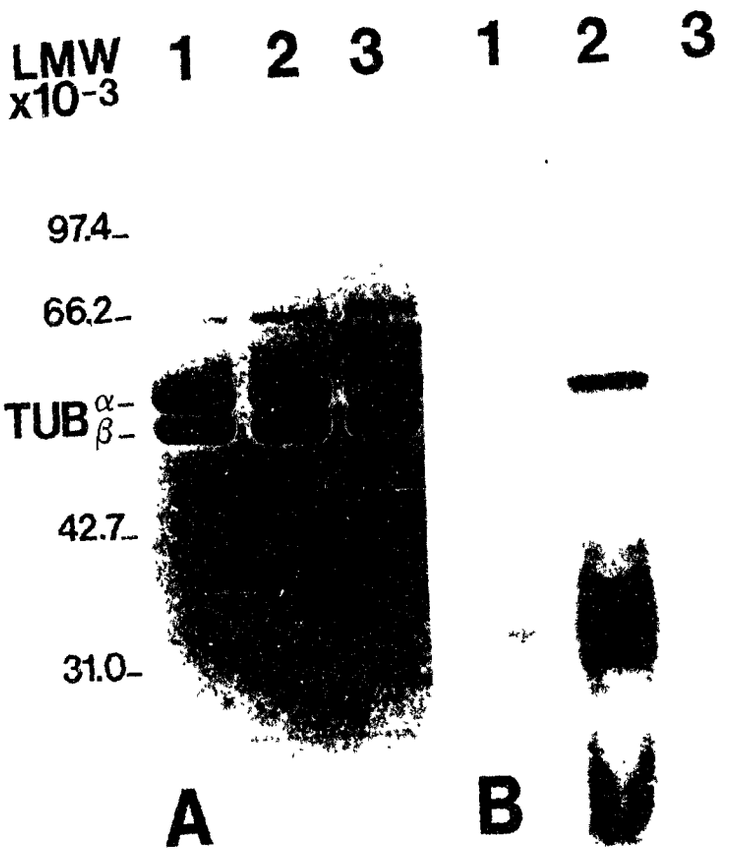


Figure 16.

Figure 17. Comparison of the amount of tubulin translated *in vitro* from *Artemia* total cytoplasmic and polysomal RNAs. Total cytoplasmic poly(A)$^+$ RNA from organisms developed 15 h (lane 1) and polysomal poly(A)$^+$ RNA from organisms developed 0 h (lane 2), 15 h (lane 3), and 24 h (lane 4) were translated *in vitro* in rabbit reticulocyte lysate, co-assembled with unlabelled 15 h *Artemia* tubulin, and electrophoresed on a 10% SDS-polyacrylamide gel as described in Materials and Methods. Five μg of RNA were translated in each case. The figure is a fluorogram of the SDS-polyacrylamide gel. The molecular masses of standards (kilodaltons) are shown on the left side of the fluorogram. α, α-tubulin; β, β-tubulin.

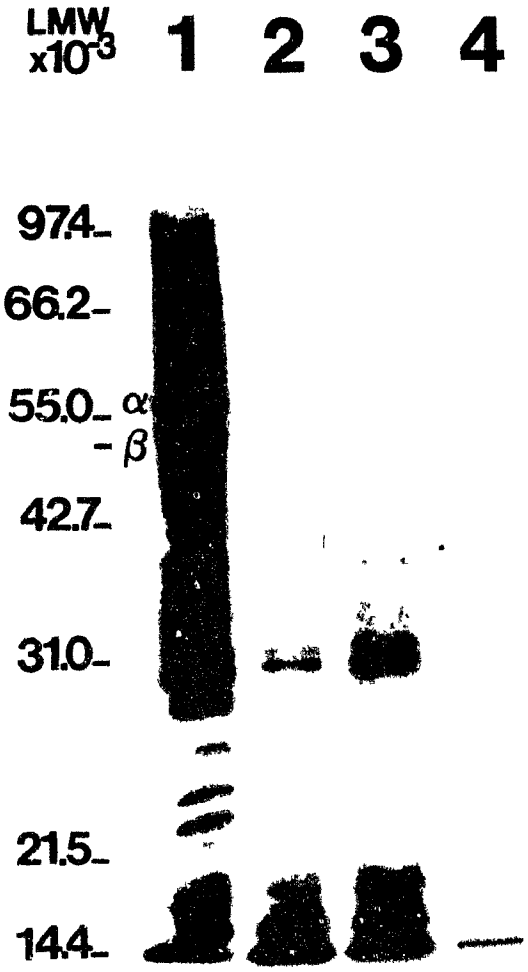


Figure 17.

reaction. In any case, assembled tubulin - unlabelled and labelled - would have passed through the cushion. The low level of tubulin translated *in vitro* from polysomal poly(A)$^+$ RNA supported the finding that very little tubulin mRNA is detectable by Northern blot analysis of the same RNA. *Artemia*, then, appears to be utilizing only a small amount of the total tubulin mRNA available during the first 24 h of post-gastrular development.

In vivo labelling of *Artemia* tubulin

In order to examine the synthesis of tubulin in *Artemia in vivo*, and as an adjunct to the analysis of *Artemia* polysomal RNA, *Artemia* were labelled *in vivo* by incubation in hatch medium containing ^{14}C-sodium bicarbonate in place of unlabelled sodium bicarbonate. Cell-free homogenates were then prepared in the usual fashion and the incorporation of ^{14}C into acid-precipitable material was determined, as shown in Table 6. Twenty-five µg of total protein from organisms developed 0 h, 15 h, and 24 h were electrophoresed on a 10% SDS-polyacrylamide gel. The Coomassie blue stained gel and the corresponding fluorogram are shown in Figure 18, panels A and B. As expected, 0 h organisms, which were only exposed to ^{14}C-bicarbonate for several minutes, yielded a homogenate containing no labelled proteins. However, cell-free homogenates from organisms exposed to ^{14}C-bicarbonate for .5 h and 24 h contained many different labelled proteins. In order

Table 6. Incorporation of ^{14}C into acid precipitable material in *Artemia* cell-free homogenates.

Development time (h)	Protein concentration in cell-free homogenates (mg/mL)	Acid-precipitable cpm/mg protein in cell-free homogenate
0	4.0	2.5×10^3
15	8.2	1.3×10^5
24	7.4	1.8×10^5

Artemia were incubated in the presence of ^{14}C-sodium bicarbonate for 0, 15, or 24 h prior to the preparation of cell-free homogenates. The incorporation of ^{14}C into acid-precipitable material was determined as described in Materials and Methods.

Figure 18. abelling of *Artemia* proteins *in vivo*. *Artemia* were ..ted in the presence of ^{14}C-sodium bicarbonate for 0 h (lane 1), 15 h (lane 2), or 24 h (lane 3) prior to preparation of cell-free homogenates. Twenty-five µg of total protein were run on 10% SDS-polyacrylamide gels (panels A and B). Tubulin in 200 µg total protein was induced to assemble with taxol and centrifuged through 20% sucrose cushions, followed by electrophoresis (panels C and D). Panels A and C show Coomassie Blue stained gels; panels B and D show the corresponding fluorograms. Fluorograms were exposed for approximately 21 d. The molecular masses of standards (kilodaltons) are shown on the left side of the figure.

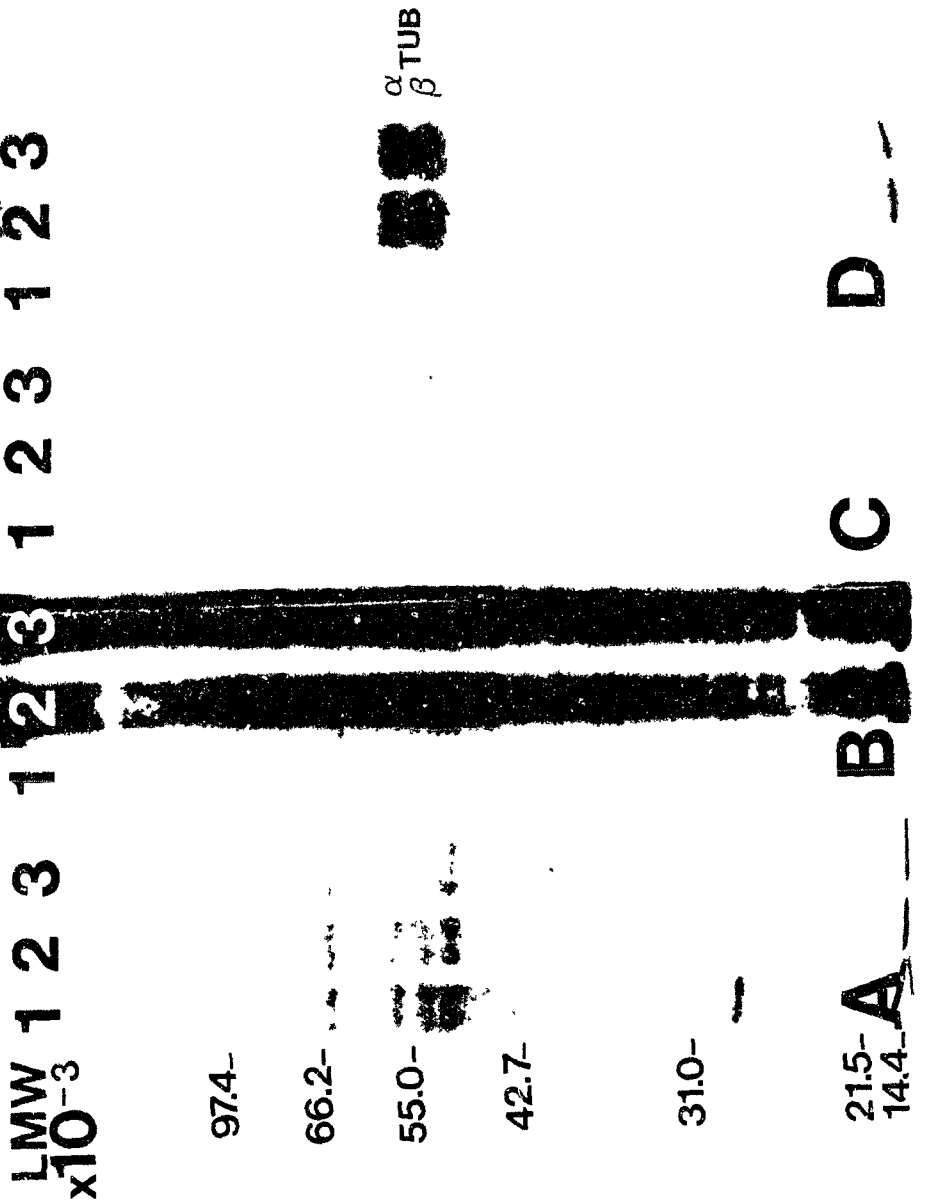

LMW
x10⁻³
1 2 3
97.4
66.2
55.0
42.7
31.0
21.5
14.4
A
1 2 3
B
1 2 3
C
1 2 3
D
α TUB
β TUB

Figure 18.

that the tubulins could be examined, it was necessary to purify them from the rest of the labelled proteins in the homogenates. To this end, taxol-induced polymerization followed by centrifugation through sucrose cushions was employed, similar to the technique used in the analysis of *in vitro* translation products. No unlabelled *Artemia* tubulin was added to the assembly reactions. However, in order for the bands to be resolved in a reasonable length of time, 100-200 μg total protein were used in the assembly reactions. Figure 18, panels C and D, shows the Coomassie blue stained gel and corresponding fluorogram of assembly-purified *in vivo* labelled *Artemia* tubulin. No labelled tubulin was seen in 0 h samples, but 15 h and 24 h samples contained one labelled α-tubulin band and one labelled β-tubulin band. In an attempt to elucidate their isotype composition, tubulins labelled in vivo and purified by taxol-induced polymerization were electrophoresed on two-dimensional gels (not shown). The small amount of tubulin present, combined with the long exposure time needed to produce visible α- and β-tubulin spots on the fluorogram, made it impossible to determine the number of isotubulins synthesized *in vivo*.

Analysis of post-translational modifications to purified *Artemia* tubulin

Artemia tubulin purified to homogeneity was blotted to nitrocellulose and probed with a panel of monoclonal and polyclonal antibodies to tubulin. The immunological reac-

Table 7. Immunological reactivity of purified *Artemia* tubulin.

purified tubulin	poly-clonal	DM1A	6-11B-1	YL1/2	anti-Glu	anti-Tyr	TU-01	KMX	DM1B	TUB2.1
0 h	n.d.	+++	++/+++	++	-	++	++	++	-	-
15 h	++	+++	++/+++	++	-	+/++	++	++	-/+	-/+
24 h	n.d.	+++	++/+++	-/+	++	-/+	++	++	-	-
24 h + inhib.	n.d.	+++	++/+++	+	+/++	+	++	++	n.d.	n.d.
bovine brain tubulin	++/+++	n.d.	++	++	++/+++	+++	+++	++/+++	-	++/+++

-, no reaction
+, weak reaction
++, moderate reaction
+++, strong reaction
n.d., not done

tivities of *Artemia* tubulin to the various antibodies are summarized in Table 7. An early finding was that anti-Glu, the polyclonal antibody specific for detyrosinated (Glu-) α-tubulin, showed no reaction with 0 h or 15 h α-tubulin, but reacted quite strongly with 24 h α-tubulin. A parallel finding was that YL1/2, the monoclonal antibody specific for tyrosinated (Tyr-) α-tubulin, reacted with 0 h and 15 h *Artemia* α-tubulin, but showed a significant drop in the intensity of the reaction with 24 h α-tubulin. The polyclonal antibody anti-Tyr, also specific for tyrosinated α-tubulin, showed a similar decrease in the intensity of the reaction with 24 h α-tubulin (Fig.23, panel G).

The terminal tyrosine of tubulin is removed by the action of carboxypeptidase, and it is known that a tubulin-specific carboxypeptidase co-purifies with tubulin from brain (Arce and Barra, 1983). Further, the action of tubulin-specific carboxypeptidase may be mimicked by the action of general carboxypeptidases present in cell extracts (Modesti and Bulinski, 1989) such as may be found in homogenates of *Artemia* developed for 24 h. Such *Artemia* are free-swimming nauplii with a developing gut, and therefore contain digestive enzymes that are not found in 0 h or 15 h *Artemia*. It was possible, then, that in addition to detyrosinated α-tubulin produced *in vivo*, detyrosinated α-tubulin may have been produced as a result of the action of co-purifying carboxypeptidase(s) during the purification of 24 h *Artemia*

Figure 19. Carboxypeptidase A detyrosination of *Artemia* tubulin. 2.5 μg of purified tubulin from organisms developed 15 h were electrophoresed on 10% SDS-polyacrylamide gels and transferred to nitrocellulose. Lanes 2 and 4 were treated with carboxypeptidase A; lanes 1 and 3 received identical treatment but were not exposed to carboxypeptidase A. Lanes 1 and 2 were immunostained with the anti-tyrosinated α-tubulin antibody YL1/2; lanes 3 and 4 were immunostained with the anti-detyrosinated α-tubulin antibody anti-Glu. Only the tubulin region of the blots is shown, as no other bands were visible on the blots. TUB, tubulin.

Figure 19.

tubulin. In order to demonstrate the vulnerability of *Artemia* tubulin to detyrosination by carboxypeptidase, 15 h tubulin blotted to nitrocellulose was exposed to bovine pancreatic carboxypeptidase A, type I, following the protocol of Gundersen *et al.* (1987). The carboxypeptidase had been treated with PMSF to eliminate any contaminating trypsin and chymotrypsin activity. Figure 19 shows the complete elimination of YL1/2 reactivity with a simultaneous appearance of reactivity with the anti-Glu antibody, demonstrating that: a) *Artemia* tubulin is sensitive to the action of a detyrosinating enzyme and b) YL1/2 and anti-Glu reacted specifically with tyrosinated and detyrosinated α-tubulin, respectively, on the Western blots.

An attempt was made to inhibit the action of the carboxypeptidase(s) proposed to be detyrosinating 24 h *Artemia* tubulin by including the general carboxypeptidase A inhibitors hydrocinnamic acid (Kobayashi and Matsumoto, 1982) and 1,10-phenanthroline (Deanin et al., 1980) during the purification of 24 h tubulin, in addition to the proteolytic inhibitors routinely used. Tubulin so purified was probed with the anti-Glu and YL1/2 antibodies, in parallel with 0 h, 15 h, and 24 h tubulin purified in the absence of carboxypeptidase inhibitors (Fig.20). Weak reaction of anti-Glu with 24 h β-tubulin, an occasional occurrence, may have been due to alteration of the β-tubulin by carboxypeptidase as this reaction was eliminated by the use of carboxypeptidase

Figure 20. The effect of carboxypeptidase A inhibitors during tubulin purification. Tubulin purified from organisms developed 0 h (lane 1), 15 h (lane 2), 24 h (lane 3), and tubulin purified in the presence of 1,10-phenanthroline and hydrocinnamic acid from organisms developed 24 h (lane 4) were electrophoresed on 10% SDS-polyacrylamide gels and transferred to nitrocellulose. One μg of tubulin was loaded per lane. Blotted protein was probed with anti-detyrosinated α-tubulin monoclonal antibody anti-Glu (panels A and B) and the anti-tyrosinated α-tubulin monoclonal antibody YL1/2 (panels C and D) using both the horse radish peroxidase method (panels A and C) and the alkaline phosphatase method (panels B and D). TUB, tubulin.

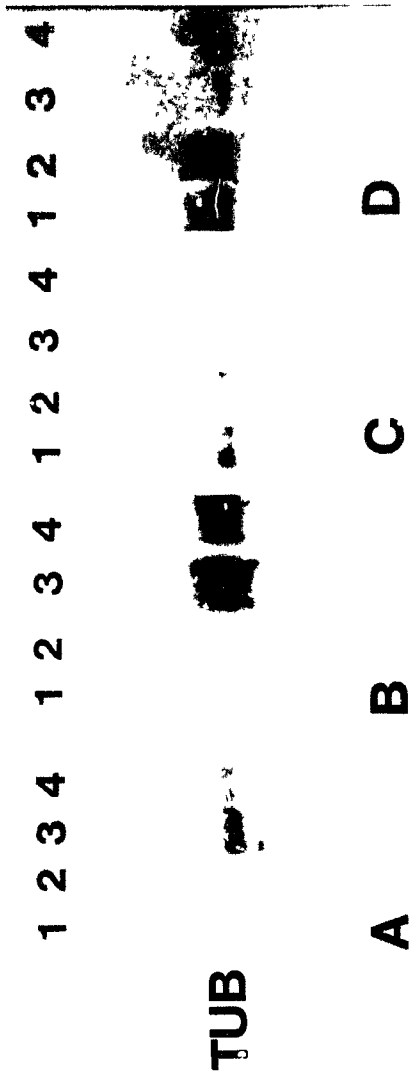


Figure 20.

inhibitors. A similar cross-reaction has been observed on sea urchin β-tubulin (Gurdersen and Bulinski, 1986). The carboxypeptidase inhibitors had a small effect on the immunoreactivity of 24 h α-tubulin, but the level of tyrosinated α-tubulin was lower and detyrosinated α-tubulin higher than was seen in 0 h and 15 h tubulin. It appeared that 24 h α-tubulin was being predominantly modified by a carboxypeptidase which was not sensitive to the action of general inhibitors. However, subsequent Western blots were performed with 24 h tubulin prepared in the presence of the carboxypeptidase inhibitors in order to diminish as much as possible the influence of non-specific carboxypeptidase artifacts on immunostaining results.

To demonstrate that 24 h tubulin - prepared in the absence and presence of general carboxypeptidase inhibitors - was still intact despite the action of modifying enzymes, it was compared to tubulin from organisms developed 0 h and 15 h on isoelectric focusing and two-dimensional gels (Figs.21 and 22, respectively). Electrophoretic mobility and isoform distribution were the same in all cases. An increased amount of stained material, which may be degraded tubulin, appears to be present on isoelectric focusing gels of 24 h tubulin prepared with or without carboxypeptidase inhibitors. The isoform pattern of tubulin, however, appears the same as that from organisms developed 0 h or 15 h.

Figure 21. Isoelectric focusing of *Artemia* tubulin. Thirty μg of purified *Artemia* tubulin from organisms developed 0 h (A), 15 h (B), 24 h (C), and tubulin purified in the presence of 1,10-phenanthroline and hydrocinnamic acid from organisms developed 24 h (D) were electrophoresed on 4% polyacrylamide-9 M urea isoelectric focusing gels. The pH range of the gels was 4.8-6.8. Gels were stained with Coomassie Blue. The extreme acidic and basic ends of the gels are not shown, and the pH range is indicated on the left side of the figure. The α- and β-tubulins are also indicated on the left side. The β-isotubulins do not resolve on these gels. The arrow indicates a minor α-isotubulin.

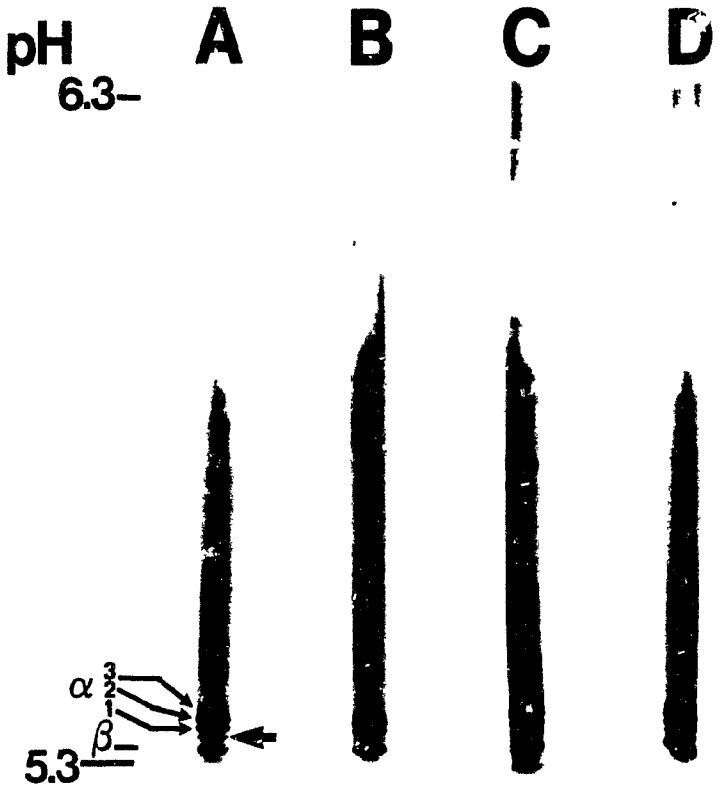


Figure 21.

Figure 22. Two-dimensional gel electrophoresis of *Artemia* tubulin. Thirty µg of purified *Artemia* tubulin from organisms developed 0 h (A), 15 h (B), 24 h (C), and tubulin purified in the presence of 1,10-phenanthroline and hydrocinnamic acid from organisms developed 24 h (D) were analysed by isoelectric focusing in one direction (pH) and SDS-polyacrylamide gel electrophoresis in the other direction (SDS). The pH decreased left to right, from approximately 5.7 to 5. Only the tubulin region of the Coomassie Blue stained gels is shown.

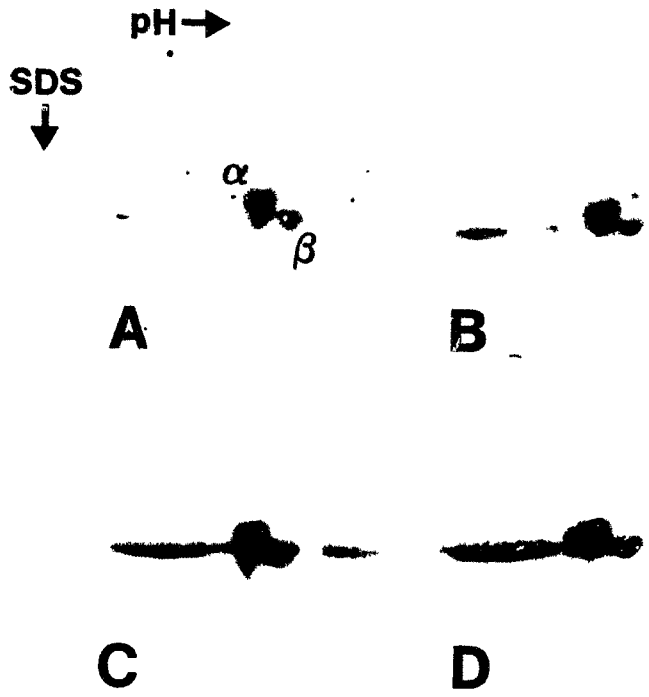


Figure 22.

Figure 23. Immunostaining of *Artemia* tubulin. One µg of purified tubulin from organisms developed 0 h (lane 1), 15 h (lane 2), and 24 h (lane 3) was electrophoresed on 10% SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with a panel of antibodies to tubulin using either the alkaline phosphatase method (panels A-E, H) or the horseradish peroxidase method (panels F and G). The antibodies used were: panel A, polyclonal anti-tubulin; panel B, DM1A; panel C, TU-01; panel D, KMX; panel E, 6-11B-1; panel F, YL1/2; panel G, anti-Tyr; panel H, anti-Glu.

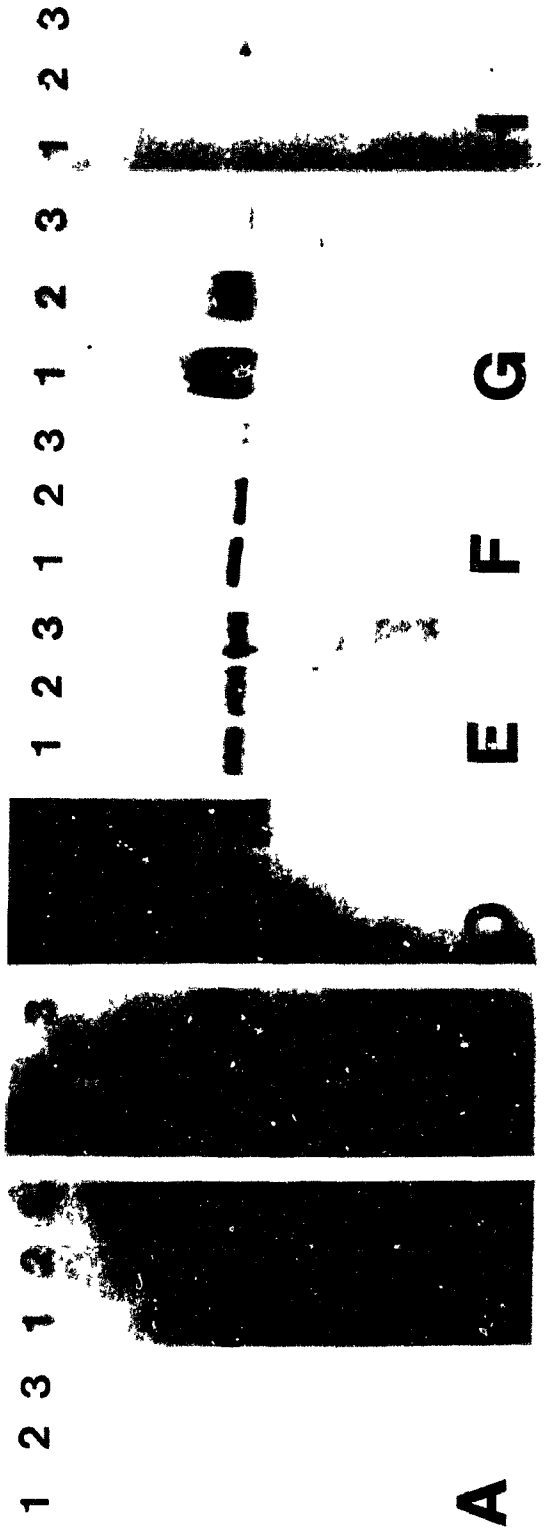


Figure 23.

Figure 24. Two-dimensional resolution of the *Artemia* acetylated α-tubulin isoform. Thirty μg of *Artemia* tubulin from organisms developed 15 h were resolved into isoforms by isoelectric focusing in one direction (pH) and SDS-polyacrylamide gel electrophoresis in the other direction (SDS), followed by transfer to nitrocellulose. The pH decreased left to right, from approximately 5.7 to approximately 5.2. Blotted tubulin was probed with anti-tubulin antibodies using the horseradish peroxidase immunostaining method. A, Coomassie Blue-stained gel; B, blot probed with the anti-acetylated α-tubulin monoclonal antibody 6-11B-1; C, blot probed with the general anti-α-tubulin monoclonal antibody TU-01. Only the tubulin region of the gel and blots is shown, as no other proteins were visible. The locations of the α- and β-tubulins are indicated. Arrow, the acetylated α-tubulin isoform.

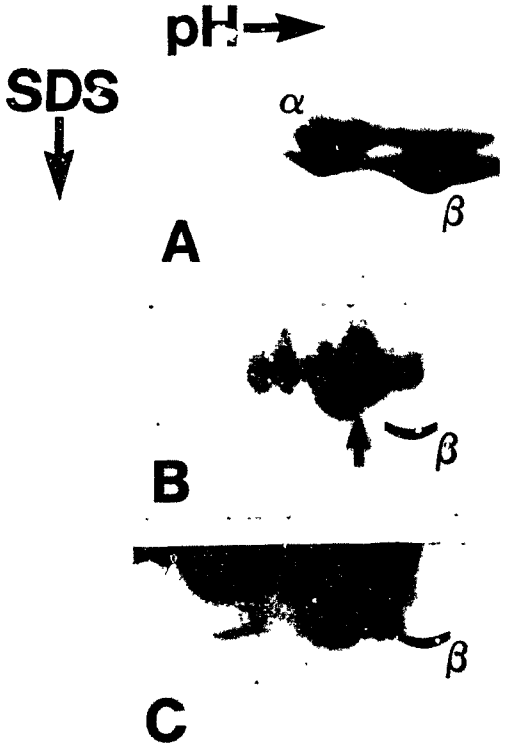


Figure 24.

The monoclonal antibody 6-11B-1, specific for acetylated α-tubulin gave a positive reaction with *Artemia* α-tubulin (Fig.23, panel E). The intensity of the reaction was equivalent for 0 h, 15 h, and 24 h tubulin, indicating an apparently constant amount of acetylated α-tubulin in *Artemia* during the first 24 h of post-gastrular development. On two-dimensional blots (Fig.24) acetylated α-tubulin localized to the most acidic α-isotubulin spot.

The general anti-α-tubulin monoclonal antibodies DM1A and TU-01 both reacted with *Artemia* α-tubulin, and the reactions were of equal intensity with tubulin from organisms developed 0 h, 15 h, or 24 h (Fig.23, panels B and C). In contrast, the general anti-β-tubulin antibodies DM1B and TUB2.1 showed very slight, variable reaction or no reaction with *Artemia* β-tubulin from any time point. However, the monoclonal antibody KMX, also a general anti-β-tubulin antibody, did react with *Artemia* β-tubulin in 0 h, 15 h, and 24 h samples (Fig.23, panel D). The results of immunostaining with anti-α- and β-tubulin antibodies indicated that the overall amount of tubulin remains constant in *Artemia* during the first 24 h of post-gastrular development.

Analysis of post-translational modifications to tubulin in cell-free homogenates

Cell-free homogenates were prepared from *Artemia*, blotted to nitrocellulose, and probed with a panel of anti-tubulin antibodies, as was done for purified tubulin. In

Figure 25. One-dimensional gel electrophoresis of *Artemia* cell-free homogenates. Cell-free homogenates were prepared from organisms developed 0 h (lane A), 15 h (lane B), 18 h/Stage 1 (lane C), 20 h (lane D), 22.5 h (lane E), 24 h (lane 5), 33 h/Stage 2 (lane G), and 43 h/Stage 3 (lane H). Twenty-five µg total protein were loaded per lane. Homogenates were electrophoresed on 10% SDS-polyacrylamide gels. The figure shows the Coomassie Blue stained gel. The molecular masses of standards (kilodaltons) are shown on the left side of the figure.

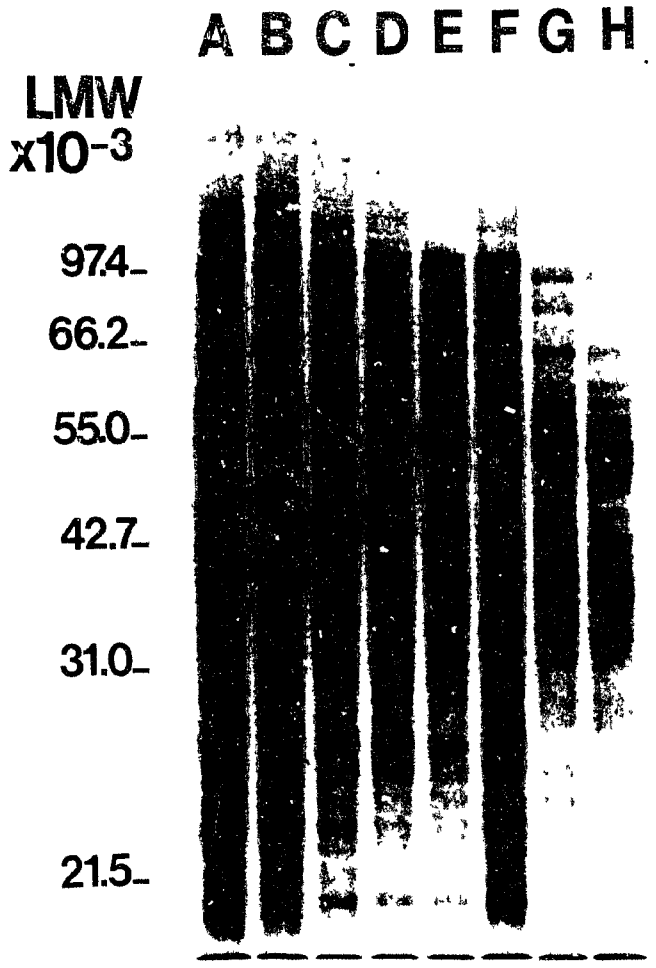


Figure 25.

addition to the three usual development times of 0 h, 15 h, and 24 h, additional homogenates were prepared from organisms developed 17 h (instar 1), 20 h, and 22.5 h. Between 15 h and 24 h of development, the organisms are hatching into actively swimming nauplii, and are developing a functional gut, so it seemed possible that changes in tubulin post-translational modifications were occurring at this time. Further, the lack of lengthy purification procedures during preparation of cell-free homogenates reduced the potential for adventitious modifications to tubulin. A Coomassie blue stained gel of the six cell-free homogenates examined is shown in Figure 25 (first six lanes).

The general anti-α-tubulin antibody DM1A and the general anti-β-tubulin antibody KMX were used together to reveal essentially constant levels of *Artemia* α- and β-tubulin (Fig.26, panel A, first six lanes). Both acetylated α-tubulin (panel B, first six lanes) and tyrosinated α-tubulin (panel C, first six lanes), visualized using the antibodies 6-11B-1 and YL1/2 respectively, appeared to be relatively constant in amount. In contrast to the results obtained with purified tubulin, immunostaining of cell-free homogenates did not show a complete loss of tyrosinated α-tubulin in 24 h samples, nor was a large amount of detyrosinated α-tubulin visible (Fig.26, panels C and D, first six lanes). The antibody anti-Glu demonstrated an apparently low level of detyro-sinated α-tubulin, with the first evidence of this

Figure 26. Immunostaining of *Artemia* cell-free homogenates. *Artemia* cell-free homogenates from organisms developed (from left to right) 0 h, 15 h, 18 h/Instar 1, 20 h, 22.5 h, 24 h, 33 h/Instar 2, and 43 h/Instar 3, were electrophoresed on 10% SDS-polyacrylamide gels (25 μg total protein/lane), transferred to nitrocellulose, and probed with anti-tubulin antibodies using the alkaline phosphatase method. Panel A, general anti-α-tubulin monoclonal antibody DM1A and general anti-β-tubulin monoclonal antibody KMX; panel B, anti-acetylated α-tubulin monoclonal antibody 6-11B-1; panel C, anti-tyrosinated α-tubulin monoclonal antibody YL1/2; panel D, anti-detyrosinated α-tubulin antibody anti-Glu. The positions of the α- and β-tubulins are indicated. tub, tubulin.

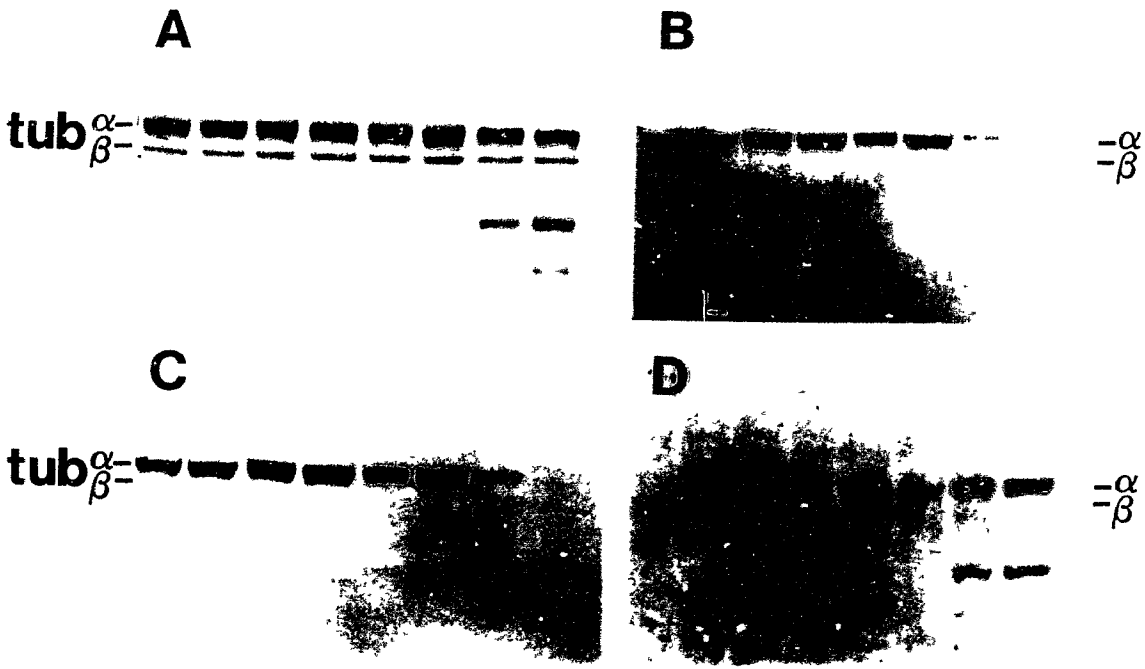


Figure 26.

isoform visible at 20 or 22.5 h. It was essential to use the alkaline phosphatase immunostaining method in this case, as the less sensitive horseradish peroxidase method did not give a visible reaction with Glu tubulin in cell-free homogenates. In the case of other antibodies used, the results were the same regardless of the immunostaining method employed.

The post-translational modifications examined on Western blots in Figure 26 were more closely analysed by scanning photopositives of the blots with a Bio-Rad video densitometer. The data from representative scans are presented, rather than cumulative data from numerous scans, as the overall intensity of immunostaining varied somewhat from one blot to another. The relationship between samples on the same blot, however, was consistent in all cases. An arbitrary figure for the amount of tubulin in the homogenates was obtained by adding together the values for the intensity of DM1A and KMX staining (in OD x mm) for each sample as provided by the Bio-Rad 1D-Analyst software. For each of the other antibodies used, the value for the inten-sity of the staining of each homogenate was divided by the overall tubulin value for that homogenate. In this way a value for the level of each post-translationally modified form of α-tubulin in relation to the total amount of tubulin was obtained. No quantitative comparison was made between the separate blots, as the affinity of each antibody for particular epitopes in *Artemia* is not known. The amount of

Figure 27. Fluctuation in the levels of post-translationally modified tubulins over development. Cell-free homogenates were blotted to nitrocellulose and immunostained using the alkaline phosphatase method. Photopositives of the blots were scanned using a Bio-Rad model 620 video densitometer. Values for the relative amount of acetylated (6-11B-1, ●——●), tyrosinated (YL1/2, ▲——▲), and detyrosinated (anti-Glu, ■——■) were obtained as described in Results.

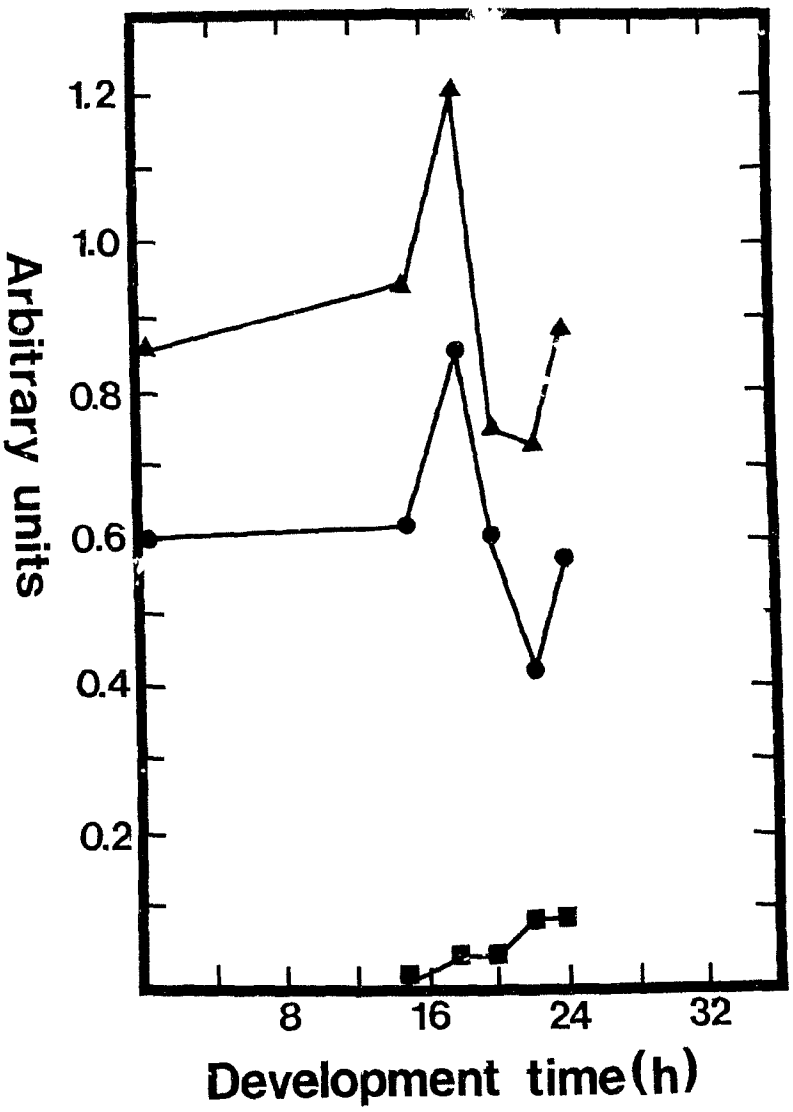


Figure 27.

total tubulin remained essentially constant over the first 24 h of post-gastrular development. However, as seen in Figure 27, some fluctuation was seen in the amounts of acetylated, tyrosinated, and detyrosinated α-tubulin. Increases in the levels of acetylated and tyrosinated α-tubulins were seen in homogenates from organisms developed between 15 h and 24 h, which could possibly correlate with physiologic events in the organism. The trend of these data, however, is of a slight decline in the levels of tubulin acetylation and tyrosination in *Artemia* during the first 24 h of development, with a roughly simultaneous increase in the level of detyrosinated α-tubulin. The immunological reactivities of the tubulin in cell-free homogenates are summarized in Table 8.

A preliminary survey was made of the immunoreactivity of 33 h (instar 2) and 43 h (instar 3) *Artemia* larvae. The levels of α- and β-tubulin (Fig.26, panel A, last two lanes) showed a slight decrease from levels seen in 0 h to 24 h organisms. The levels of acetylated and tyrosinated α-tubulin (panels B and C respectively, last two lanes) showed a much more dramatic decline. Conversely, detyrosinated α-tubulin was considerably more abundant in 33 h and 43 h cell-free homogenates than in homogenates from earlier points in development. However, there is evidence of proteolytic breakdown of the proteins in 33 h and 43 h cell-free homogenates, as seen by the lighter Coomassie blue staining in Fig. 25, lanes G and H. These findings must be interpreted

cautiously, particularly in light of the finding by Rafiee *et al.* (1986b) that tubulin is very difficult to isolate from organisms developed approximately 36 h due to the high content of degradative enzymes in the *Artemia* gut.

Table 8. Immunological Reactivity of *Artemia* Cell-Free Homogenates

Development time	anti-tubulin	DM1A	6-11B-1	YL1/2	anti-Glu	anti-Tyr	TU-01	KMX	DM1B	TUB2.1
0 h	++	++	++	++	-	++	++	++	-	-
15 h	++	++	++	++	-	++	++	++	-	-
Stage 1 (17.5 h)	n.d.	++	++	++	-	++	++	++	n.d.	n.d.
20 h	n.d.	++	++	++	+	n.d.	n.d.	++	n.d.	n.d.
22.5 h	n.d.	++	++	++	+	n.d.	n.d.	++	n.d.	n.d.
24 h	++	++	++	+/++	+/++	++	++	++	-	-
bovine brain tubulin	++/+++	n.d.	++	++	++/+++	+++	+++	++/+++	-	++/+++

-, no reaction
+, weak reaction
++, moderate reaction
+++, strong reaction
n.d., not done

DISCUSSION

Artemia tubulin mRNA

In this study the synthesis and post-translational modification of *Artemia* tubulin were investigated, in order to elucidate the source(s) of isotubulin diversity in this organism. As was shown by Langdon *et al.* (1990), *Artemia* appear to possess a limited complement of tubulin genes. Only one α-tubulin gene and, at most, a small number of β-tubulin genes have been detected in *Artemia*, a finding noteworthy in light of the complexity of tubulin gene families in other organisms (reviewed by MacRae and Langdon, 1989). Most other metazoan organisms have a number of α- and β-tubulin genes, but *Artemia* has a gene family comparable in size to that of far less complex organisms such as yeast and *Tetrahymena*. However, *Artemia* contain three major α-tubulins and two major β-tubulins.

In order to examine the origin of isotubulin diversity in *Artemia*, tubulin mRNA was examined on Northern blots by hybridization to the cloned *Drosophila* α- and β-tubulin genes contained in plasmids pDmTα1 and DTB2 respectively. While *Artemia* tubulin gene probes would have been preferable, the tubulin genes of *Artemia* have not yet been cloned. The *Drosophila* tubulin gene clones, however, represent well conserved genes which would be expected to hybridize with most other tubulin genes. Tubulin genes show a high degree of sequence conservation, particularly in regions encoding

functionally essential domains on the tubulin molecule (Ginzberg *et al.*, 1985). The cloned *Drosophila* tubulin genes hybridized at high stringency to *Artemia* DNA, showed no cross-reaction between *Artemia* α- and β-tubulin genes, and were used to establish the minimum size of the *Artemia* tubulin gene family (Langdon *et al.*, 1990). When used for analysis of *Artemia* tubulin mRNA in total cytoplasmic RNA - which is composed of the cytoplasmic and polysomal RNA pools - a single size class of 1.9 kB for both α- and β-tubulin mRNA was revealed. The size and abundance of the mRNA were constant for 0 h, 15 h, and 24 h RNA preparations, and the tubulin mRNA was found primarily in the poly(A)$^+$ RNA fraction. The size of the *Artemia* tubulin mRNA is comparable with that of numerous other organisms, most tubulin mRNAs falling in the range 1.7 to 2.4 kB.

As a control, it would have been useful to re-probe the Northern blots with an *Artemia* cDNA probe for a housekeeping gene. Unfortunately, as research into the molecular genetics of *Artemia* is still in the early stages, no housekeeping genes suitable for use as a cDNA probe have yet been determined. As a further control, the level of ribosomal RNA in the RNA preparation could have been examined, as changes in the level of rRNAs would influence the amounts of other RNAs present on the Northern blots. However, the formaldehyde gels were stained with ethidium bromide and examined under ultraviolet light and visual inspection did not reveal

noticeable changes in the levels of rRNA present over the first 24 h of *Artemia* development.

The hybridization signals obtained from the ^{32}P-labelled plasmid DTB2 were consistently less intense than those obtained using plasmid pDmTα1, despite comparable specific activities of the labelled plasmids. The reason for this could be a slightly lower degree of sequence similarity between the *Drosophila* β-tubulin gene probe and *Artemia* β-tubulin mRNA than between the *Drosophila* α-tubulin gene probe and *Artemia* α-tubulin mRNA.

It is possible that the individual bands of α- and β-tubulin mRNA on Northern blots are composed of more than one tubulin mRNA of the same size, particularly in the case of the β-tubulin mRNA. Such a situation could arise if several nearly identical tubulin genes were present and expressed in the *Artemia* genome, rather than a single α-tubulin gene and a very small number of β-tubulin genes. To further analyse the *Artemia* tubulin mRNAs then, total cytoplasmic poly(A)$^+$ RNA was translated *in vitro* in a rabbit reticulocyte lysate system. In accordance with findings from other laboratories (Slegers and Aerden, 1988; James and Tata, 1980; Slegers *et al.*, 1977, Grosfeld and Littauer, 1976a), *in vitro* translation of poly(A)$^+$ RNA from dormant gastrulae (0 h) yielded lower levels of ^{35}S-methionine incorporation into acid-precipitable material than did RNA from organisms developed for 15 h or 24 h. Inhibition of translation of mRNA from

dormant gastrulae, which exists *in vivo* as mRNP particles, may be caused by the combined effects of phosphorylating enzymes associated with cytoplasmic mRNP, which increase the poly(A) binding capacity of mRNP proteins, and a small RNA associated with mRNP which abolishes the initiation of translation (Slegers and Aerden, 1988; Van Hove *et al.*, 1985). In general, however, *Artemia* poly(A)$^+$ RNA translated well *in vitro*, yielding a large number of different labelled polypeptides. Preliminary work in other laboratories revealed single α- and β-tubulins on one-dimensional gels of unfractionated *Artemia* poly(A)$^+$ RNA *in vitro* translation products (Grosfeld and Littauer, 1976a). In an effort to better resolve the *Artemia* tubulins synthesized *in vitro* in this study, they were purified by co-assembly with unlabelled *Artemia* tubulin and centrifugation through sucrose cushions. Taxol and unlabelled tubulin were added in order to ensure that the *Artemia* tubulin synthesized *in vitro* polymerized efficiently, as tubulin does not polymerize at very low concentrations. Further, the co-assembly reaction helps select for complete or nearly complete tubulin molecules. This approach has been used in the study of *Naegleria* tubulin translated *in vitro* (Lai *et al.*, 1979), and is unlikely to "miss" isoforms as even quite divergent or chimeric tubulins will co-polymerize (Baker *et al.*, 1990; Prescott *et al.*, 1989; Gu *et al.*, 1988; Hussey *et al.*, 1987; Lewis *et al.*, 1987; Bond *et al.*, 1986). Based on the criteria of co-

assembly and co-migration of unlabelled *Artemia* tubulin with two labelled polypeptides translated *in vitro*, I feel confident that the polypeptides are in fact *Artemia* α- and β-tubulin. When separated in two dimensions, a single spot for α- and a maximum of two spots for β-tubulin were obtained. Single α- and β-tubulin spots were demonstrated by Grosfeld and Littauer when *in vitro* synthesized proteins were analyzed on two-dimensional gels (Grosfeld and Littauer, 1976a). However, this earlier work was based on analysis of unfractionated *in vitro* translation products, and the identification of electrophoretic spots as tubulin was based solely on migration patterns. In the present study, *in vitro* translation was apparently more successful, as such a large number of labelled proteins were obtained as to make analysis of the tubulins impossible without purifying them by co-assembly. The possibility that the one α- and one or two β-tubulin spots are composed of more than one electrophoretically identical tubulin arising from two or more α- and/or β-tubulin mRNAs of the same size, as is the case in *Physarum* (Birkett *et al.*, 1985), cannot be ruled out. The simplest interpretation of these data, however, is that a single α-tubulin arises from translation of a single mRNA. Two β-tubulins likely arise from two mRNAs of the same length, which therefore appear as a single band on Northern blots.

Further analysis of *in vitro* translated *Artemia* tubulins by immunoprecipitation was attempted, as was *in vitro* trans-

lation of hybrid-selected *Artemia* tubulin mRNAs, but neither approach yielded reproducible results.

In order to determine how much tubulin was being synthesized by *Artemia*, polysomal poly(A)$^+$ RNA was examined. The dormant *Artemia* gastrulae (0 h) contain only a small amount of polysomes, which have been proposed to be either left over from those present in the embryo prior to dessication, or to be in place to implement rapid resumption of protein synthesis once development resumes (Grosfeld and Littauer, 1976b; Clegg and Golub, 1969). Accordingly, the yield of polysomal poly(A)$^+$ RNA from dormant gastrulae was less than half of that obtained from organisms which had developed for 15 h. When polysomal poly(A)$^+$ RNAs were probed with the ^{32}P-labelled plasmid pDmTα1, only a small amount of α-tubulin mRNA was detectable in polysomal RNA at 0 h, 15 h, and 24 h. Although the hybridization signals were not detected, β-tubulin mRNA was probably present in small amounts in polysomal RNA, since β-tubulin was detected by *in vitro* translation of polysomal RNA and *in vivo* labelling of *Artemia* proteins.

The incorporation of ^{35}S-methionine during *in vitro* translation of polysomal poly(A)$^+$ RNA from dormant gastrulae was lower than for translation of polysomal poly(A)$^+$ RNA from organisms developed 15 h or 24 h, likely for the same reasons as noted for translation of 0 h total cytoplasmic poly(A)$^+$ RNA. In general, though, many ^{35}S-methionine labelled peptides were obtained, over a range of molecular weights.

Compared to the tubulin obtained by *in vitro* translation of total cytoplasmic poly(A)$^+$ RNA, however, only a small amount of tubulin was translated from polysomal poly(A)$^+$ RNA. A somewhat larger amount of tubulin appears to be translated *in vitro* from polysomal poly(A)$^+$ RNA from organisms developed 15 h than from organisms developed 0 h or 24 h. It is possible that more tubulin mRNA is present on polysomes at 15 h, compared to 0 h and 24 h of development. It is more likely, however, that the improved translation of 15 h poly(A)$^+$ RNA preparations (Table 5) accounts for the heavier tubulin bands.

In accordance with the data obtained from Northern blot analysis, *Artemia* appear to be synthesizing only a small amount of tubulin during the first 24 h of postgastrula development: there is no substantial increase in the amount of tubulin mRNA loaded onto polysomes over that already present on polysomes in dormant gastrulae. Evidently then, developing *Artemia* contain sufficient tubulin in the dormant gastrula so that large amounts need not be synthesized for use in morphogenesis, once development resumes.

The picture of tubulin synthesis in developing *Artemia* suggested by *in vitro* findings was supported by the results of *in vivo* labelling of *Artemia* proteins using ^{14}C-sodium bicarbonate. The basis for ^{14}C-labelling in *Artemia* is as follows: the developing embryo derives metabolic energy from the breakdown of stored glycogen, but high ATP requirements

are imposed by osmoregulation, particularly sodium excretion, in the saline environment of the organism (Conte and Geddes, 1988). Additional ATP is furnished via a facultative glycolytic cycle which requires a CO_2 fixation step, GDP as a cofactor, and phosphoenolpyruvate. The required CO_2 is derived from carbonate ions in the surrounding water. Experimentally, this carbonate can be labelled with ^{14}C, so that end-products of the cycle - amino acids, Kreb's cycle intermediates and their acids, and pyrimidine nucleotides - are labelled. Aspartic acid, malic acid, and alanine are the amino acids that incorporate the largest amount of ^{14}C, probably due to the fact that they show rapid turnover (Clegg, 1976).

In this study, labelling of *Artemia* proteins *in vivo* was successful. The apparent incorporation of ^{14}C into proteins in dormant gastrulae indicated by acid-precipitable counts is likely due to contaminating $H^{14}CO_3$ adhering to fragments of the cyst shell, as no labelled proteins were seen on fluorograms of 0 h cell-free homogenates. Organisms incubated for 15 h and 24 h in the presence of $H^{14}CO_3$ synthesized a large number of labelled proteins. One-dimensional gels of polymerized tubulins from *in vivo* labelled cell-free homogenates revealed α- and β-tubulin. On two-dimensional gels, single α- and β-tubulin spots were detectable. As these tubulins were synthesized *in vivo*, it is probable that the tubulin spots actually contain the same isotubulins seen on Coomassie

blue stained gels of purified tubulin, but this could not be demonstrated. The small amount of labelled tubulin present and the lengthy exposure time required to obtain the fluorograms contributed to the inability to resolve the individual isotubulins. It should be noted that relatively large amounts (200 µg total protein) of cell-free homogenates were required to obtain tubulin signals on the fluorograms in reasonable lengths of time (21 d). Taken together, the results of polysomal RNA analysis and *in vivo* labelling suggest that *Artemia* show limited synthesis of tubulin during the first 24 h of post-gastrular development.

Based on the results presented here, *Artemia* contain two pools of tubulin mRNA during the first 24 h of post-gastrular development: a small, translated pool and a larger, non-translated cytoplasmic pool. Both pools appear to be composed of the same α- and β-tubulin mRNAs, at least at the level of resolution of the experiments. The reason and mechanisms by which *Artemia* maintain such a surplus amount of untranslated tubulin mRNA are unclear. In dormant *Artemia* gastrulae a certain amount of tubulin mRNA stored as cytoplasmic or membrane-bound mRNP particles might be expected, as many mRNAs are stored in this fashion (Nilsson and Hultin, 1982; James and Tata, 1980). The situation in organisms developed 15 h and 24 h is more unusual. If the tubulin mRNA is stored from the cryptobiotic phase, one would expect some sort of mechanism to be operating in order to

protect the mRNA from enzymatic degradation. Stabilization is known to be conferred in *Physarum* tubulin mRNAs by long 3' poly(A) tracts (Green and Dove, 1988). Nonvariant histone mRNAs - which are not polyadenylated - are stabilized by particular sequences in the 5' or 3' untranslated regions (Hunt, 1988; Brawerman, 1987). In cultured mammalian cells, Cleveland and co-workers have demonstrated the existence of an autoregulatory mechanism controlling the stability of tubulin mRNA present on polysomes. Evidence for a similar mechanism has been found in sea urchin embryos (Gong and Brandhorst, 1988b). It is possible that, in *Artemia*, tubulin mRNA levels are controlled by an autoregulatory mechanism, although there is no evidence for such a mechanism at present. However, I feel it is more likely that the tubulin mRNA only becomes part of polysomes in very small quantities. The translation of ribosomal proteins in *E. coli* is regulated by a feedback mechanism (Dean and Nomura, 1980). When multiple copies of ribosomal protein genes are introduced into *E. coli*, the level of ribosomal protein mRNA increases dramatically but the level of the actual proteins rises very little. The binding of excess ribosomal protein to specific sequences at the 5' end of the ribosomal protein mRNA blocks the initiation of translation, thus preventing the accumulation of large, and possibly deleterious, amounts of the proteins. Although *Artemia* is a eukaryote, a similar mechanism can be envisaged to act in the regulation of

tubulin mRNA translation. In mouse, Lewis and Cowan (1988) found that myoblast fusion during muscle differentiation is accompanied by the induction of high levels of the divergent α-tubulin, Mα4 mRNA. However, very low levels of the Mα4 protein are found in only a subset of the myotubes, leading Lewis and Cowan to propose that Mα4 mRNA is subject to translational or post-translational regulation. The marked difference between the levels of Mα4 mRNA and Mα4 α-tubulin is similar to the situation in *Artemia*, and both cases could potentially be produced by the same mechanism. Such a mechanism could involve binding of the tubulin to its mRNA, as in the case of ribosomal proteins in *E. coli*, or could involve a different, as yet unknown, process. Further research, especially analysis of the transcription of *Artemia* tubulin genes, will help to clarify the picture of tubulin gene expression in *Artemia*. It would be interesting to examine the mRNAs for other proteins, particularly cytoskeletal proteins, to see if they, too, are translationally regulated in a similar fashion to tubulin.

Post-translational modifications to *Artemia* tubulin

Post-translational modifications to *Artemia* tubulin were analysed using a number of antibodies to tubulin. As analysis of *Artemia* tubulin mRNA *in vitro* and *in vivo* labelling with ^{14}C-bicarbonate gave no evidence for the translation of more than one α-tubulin, post-translational modifications appeared to be the likely route by which the three α-tubulins

observed in the first 24 h of post-gastrular development are produced. The idea that tubulin post-translational modifications can serve as a major source of isotubulin diversity in *Artemia* is not without precedent. The ciliate *Tetrahymena pyriformis* has only one α- and two β-tubulin genes, but eight α-isotubulins and four β-isotubulins (Barhona *et al.*, 1988). *Tetrahymena* tubulin genes contain several transcription initiation and termination sites, making it likely that some isotubulin diversity arises at the level of transcription. However, acetylation of the α-tubulins and phosphorylation of the β-tubulins occur and have been proposed to be important modes of isotubulin diversification in *Tetrahymena*. Similarly, two types of post-translational modification to tubulin have been demonstrated in this study of *Artemia* tubulin. Acetylation and detyrosination both occur on α-tubulin, and they are the only demonstrated ways in which the single α-tubulin translated *in vivo* is modified.

Immunostaining of *Artemia* tubulin - both purified and in cell-free homogenates - with the general anti-α-tubulin antibody DM1A (Blose *et al.*, 1984) gave a strong reaction with *Artemia* α-tubulin. The epitope for DM1A may be conformational or discontinuous, and appears to include amino acids 423-450 (Breitling and Little, 1986) or 415-443 (de la Vina *et al.*, 1988), very near the carboxy terminus of the tubulin molecule. This epitope is evidently present in

Artemia α-tubulin. Likewise, the anti-α-tubulin antibody TU-01 reacted well with *Artemia* α-tubulin. The epitope for TU-01 lies close to the amino terminus of the tubulin molecule, at least partly within the peptide containing residues 65-79 (Dráber *et al.*, 1989; Grimm *et al.*, 1987). TU-01 is not as general an antibody as DM1A; the α-tubulin from *Dictylostelium discoideum*, *Leishmania tropica*, and *Herpetomonas muscarum* (Grimm *et al.*, 1987; Dráber *et al.*, 1986) does not react with TU-01. It is noteworthy, then, that *Artemia* α-tubulin did react with TU-01. In contrast, general anti-β-tubulin antibodies reacted poorly with *Artemia* β-tubulin. The antibody TUB2.1 (Gozes and Barnstable, 1982) reacted very weakly with *Artemia* β-tubulin, and DM1B (Blose *et al.*, 1982) did not react at all. The antibody KMX (Birkett *et al.*, 1985) did, however, react well with *Artemia* β-tubulin. The KMX antibody was raised against *Physarum* β-tubulin, but the precise epitope is not known. *Artemia* β-tubulin may be more divergent than *Artemia* α-tubulin, then, as it reacts less well with general anti-β-tubulin antibodies than does α-tubulin with general anti-α-tubulin antibodies. On Northern blots, the less intense hybridization signals obtained using the *Drosophila* β-tubulin gene clone probe, compared to those obtained with the *Drosophila* α-tubulin clone, provide additional evidence for *Artemia* β-tubulin being potentially more divergent than *Artemia* α-tubulin. *Artemia* has been termed a "fast clock" organism; that is, it

has a high rate of mutation (Baxter-Lowe *et al.*, 1989). Study of *Artemia* proteins, including tubulin, can therefore aid in demonstrating those domains which are functionally essential and therefore conserved, and those which are divergent as a result of being non-essential. The epitopes for DM1B and TUB2.1 may not lie in functionally essential domains of β-tubulin and are therefore not conserved in *Artemia*, whereas the KMX epitope may lie in a well-conserved domain, such as tubulin, MAP, or GTP binding sites.

The presence of acetylated α-tubulin was revealed in *Artemia* through the use of the antibody 6-11B-1 (Piperno and Fuller, 1985). The site of acetylation on the α-tubulin molecule is amino acid 40, a lysine (L'Hernault and Rosenbaum, 1985; LeDizet and Piperno, 1987). Acetylation - which occurs primarily on polymerized tubulin - causes α-tubulin to focus to a more acidic position on isoelectric focusing gels, as revealed by the position of the acetylated α-tubulin isoform on two-dimensional gels of *Artemia* tubulin. While other anti-α-tubulin antibodies stained the whole group of three α-isotubulins, 6-11B-1 stained only the most acidic α-tubulin. Densitometric analysis of the immunostaining of *Artemia* cell-free homogenates with 6-11B-1 revealed a slight decline in the amount of acetylated α-tubulin over the first 24 h of development. A more dramatic decline to very low levels was seen in the preliminary data for 33 h and 43 h cell-free homogenates. It was shown previously during

attempts to purify tubulin from organisms developed for 36 h, that there is a high content of degradative gut enzymes in the nauplius (Rafiee *et al.*, 1986b; Rafiee and MacRae 1986). Results concerning tubulin post-translational modification in 33 h and 43 h cell-free homogenates must therefore be interpreted with caution, especially in light of recent immunofluorescence data demonstrating the presence of appreciable amounts of acetylated α-tubulin in 33 h and 43 h nauplii (MacRae *et al.*, 1990; MacRae *et al.*, in preparation).

The function of acetylated α-tubulin is not clear. It has, in many cases, been observed in a subset of stable microtubules (Dráber *et al.*, 1989; dePennart *et al.*, 1988; Khawaja *et al.*, 1988; Wolf *et al.*, 1988; Sasse *et al.*, 1987; Greer *et al.*, 1985) and microtubules rich in acetylated α-tubulin tend to be resistant to the action of the microtubule depolymerizing drugs colchicine and nocodazole (Wolf *et al.*, 1988; Piperno *et al.*, 1987). However, acetylation is not thought to be the cause of stability, but rather is likely to reflect stability conferred in some unknown fashion. Non-acetylated microtubules artificially stabilized with taxol become acetylated in some cultured cells, demonstrating that acetylation is secondary to the stabilizing mechanism itself (Webster and Borisy, 1989; Bulinski *et al.*, 1986a). Microtubule associated proteins have been proposed to be one way in which microtubules might be stabilized (Falconer *et al.*, 1989; Bulinski *et al.*, 1988a). In some cells, or at

certain points in development, acetylated α-tubulin is absent, due to either an absence of acetyltransferase (Wolf *et al.*, 1988) or possibly a microtubule turnover rate too rapid to permit acetylation to occur (Bulinski *et al.*, 1988a; Piperno *et al.*, 1987). In any case, acetylated α-tubulin is not required for cell viability and conclusions regarding the function of this form of post-translational modification are difficult to draw as yet.

Tyrosinated (Tyr) and detyrosinated (Glu) α-tubulin were present in *Artemia*. Unlike the results obtained with general and anti-acetylated tubulin antibodies, however, the data obtained for the amount of Tyr and Glu tubulin present depended very much upon whether purified tubulin or cell-free homogenates were examined. Purified tubulin showed an almost complete elimination of Tyr tubulin in 24 h samples, while Glu tubulin was abundant only in 24 h samples. Cell-free homogenates, on the other hand, showed a level of Tyr tubulin in 24 h samples not much reduced from that seen in samples from earlier times in development, and a low level of Glu tubulin. These data could be produced by the onset of synthesis of an α-tubulin bearing a terminal glutamic acid - an unusual occurrence - with a roughly simultaneous decrease in the synthesis of an α-tubulin bearing a terminal tyrosine. However, I feel it is more likely that the increase in Glu tubulin and decrease in Tyr tubulin in 24 h samples is produced in two ways: a) by one or more general - possibly

co-purifying - carboxypeptidases, and b) by a tubulin-specific carboxypeptidase acting both *in vivo*, and *in vitro* via co-purification. There are several points supporting this idea. It has been shown by Bulinski and coworkers that general carboxypeptidases present in cell extracts can detyrosinate α-tubulin (Modesti and Bulinski, 1989). Such a situation especially involving gut carboxypeptidases, can easily be envisaged to occur during *Artemia* tubulin purification. As evidence, the presence of general carboxypeptidase inhibitors during the purification of 24 h tubulin did appear to reduce the degree of detyrosination occurring on α-tubulin. The effect was, however, limited. It was also shown by Bulinski that general carboxypeptidase inhibitors are unlikely to affect tubulin-specific carboxypeptidase (personal communication). The existence of a tubulin-specific carboxypeptidase in *Artemia* is therefore indicated. This enzyme may be newly synthesized, or newly activated; modulation of tubulin-specific carboxypeptidase activity has been demonstrated during rat muscle development (Alonso *et al.*, 1988). Finally, Glu tubulin has been observed both in rapidly processed cell-free homogenates, and *in situ* in all tubulin-containing cell structures by immunofluorescence (MacRae *et al.*, in preparation), so the appearance of Glu tubulin is not purely artifactual. Tubulin-specific carboxypeptidase acts on polymerized tubulin (Arce and Barra, 1983), so a co-purifying carboxypeptidase is likely to

detyrosinate *Artemia* tubulin at the point near the end of the purification procedure, during polymerization of the tubulin at 37 °C. Prior to that point, all steps are carried out at or below 4 °C to maintain the tubulin as α/β dimers. In light of the evidence of the action of co-purifying carboxypeptidases on purified tubulin, the immunostaining of cell-free homogenates on Western blots almost certainly yielded a more realistic picture of the status of tubulin tyrosination and detyrosination during *Artemia* development.

Tubulin tyrosination/detyrosination has recently been the object of considerable study in several laboratories. As is the case with acetylated α-tubulin, however, no clear picture has yet emerged of the specific functions - if any - of Tyr or Glu tubulin. Both isoforms of α-tubulin show similar polymerization characteristics *in vitro* (Arce *et al.*, 1987; Kumar and Flavin, 1982; Raybin and Flavin, 1977), but *in vivo* microtubules rich in Tyr tubulin tend to be more dynamic than those rich in Glu tubulin (Bulinski *et al.*, 1988b; Webster *et al.*, 1987). Like acetylated α-tubulin, Glu tubulin tends to be found in stable microtubules and in differentiated cells (Gundersen *et al.*, 1989; Khawaja *et al.*, 1988; Cambray-Deakin and Burgoyne, 1987; Sasse *et al.*, 1987; Wehland and Weber, 1987). In fact, acetylated and Glu α-tubulin have been found in the same microtubules (Bulinski *et al.*, 1988a). Detyrosination does not confer stability, however, just as acetylation does not. Microtubules in

permeabilized cultured cells can be chemically detryosinated with carboxypeptidase, but they are not stabilized by the treatment (Khawaja *et al.*, 1988). Also, examples have been found of dynamic microtubules rich in Glu or acetylated α-tubulin (Sasse and Gull, 1988), and of stable microtubules rich in Tyr tubulin (Chang and Flavin, 1988; Gundersen and Bulinski, 1986), so stability and detyrosination are not necessarily concomitant. Detyrosinated α-tubulin shares a further feature with acetylated α-tubulin in that both post-translational modifications occur on polymerized tubulin (Wolf *et al.*, 1988; Bré *et al.*, 1987; Gundersen *et al.*, 1987). Of the large number of cell lines examined, all have a population of microtubules enriched in either acetylated or detyrosinated α-tubulin, or both (Khawaja *et al.*, 1988). Evidently, cells require microtubules rich in one or the other type of α-tubulin, and perhaps both α-isoforms serve similar functions, or alternatively, serve no particular function at all.

It is fairly well accepted that post-translational modifications to tubulin serve a purpose *in vivo*, whether as effectors or merely as indicators of functional specificity of the microtubules bearing the modifications. Accordingly, changes in the tyrosination/detyrosination status of α-tubulin in cells are unlikely to be random. Tight control over the amount of Tyr and Glu tubulin in polymerized and unpolymerized states has been demonstrated experimentally

(Webster *et al.*, 1987). It is therefore probable that the changes seen in post-translationally modified tubulin during *Artemia* development are not random, and may have physiological significance. In *Artemia* cell-free homogenates, Glu tubulin increases in quantity at approximately the same point in development as acetylated tubulin is decreasing, perhaps indicating that detyrosinated α-tubulin can assume the functions of acetylated α-tubulin as development proceeds. It is unusual that *Artemia* larvae grow and undergo morphogenesis in the absence of Glu tubulin for approximately the first 20 h of post-gastrular development. Glu tubulin is evidently not required for the early survival of *Artemia*. Previous to this, only the human fibroblast cell strain 356 has been shown to survive in the absence of detyrosinated α-tubulin (Khawaja *et al.*, 1988). The changes seen in the levels of acetylated and tyrosinated α-tubulin between 15 h and 24 h of development may possibly correlate with particular physiological events in the organism. Increases in both isoforms were detected in instar 1 organisms, which may indicate that larger amounts of acetylated and tyrosinated α-tubulin are required during hatching of the nauplii or, as the nauplii become free-swimming after hatching, for increased muscle activity. The smaller increase in the levels of tyrosinated and acetylated α-tubulin seen at 22.5 h may be involved with the resumption of mitosis in the nauplii, since both of these isoforms have been demonstrated in *Artemia* mitotic figures

(MacRae *et al.*, 1990; MacRae *et al.*, in preparation). The dynamics and potential functions of post-translationally modified tubulins in *Artemia* clearly merit further research.

The aim of this work was to demonstrate the source of isotubulin diversity in *Artemia* during the first 24 h of post-gastrular development, the first 17 h of which being notable in that the organism is undergoing morphogenesis from a gastrula to a free-swimming nauplius in the absence of mitosis. A single size class of mRNA was found for both α- and β-tubulin. The tubulin mRNAs appear to be translated at a low level to yield a single α- and a maximum of two β-tubulins, resolvable on two dimensional gels. Isotubulin diversity in *Artemia* is evidently provided, at least in part, by post-translational modification; acetylated and tyrosinated α-tubulin are both present in *Artemia*, but detyrosinated α-tubulin is only detectable after approximately 20 h of development. *Artemia* are complex, multicellular organisms, but can function adequately with a very limited number of tubulin genes. Further, in the absence of mitosis, *Artemia* morphogenesis is carried out despite apparently low levels of tubulin synthesis ard in the absence of detyrosinated α-tubulin. These findings are noteworthy in light of the fact that protein synthesis is essential to early post-gastrular development in *Artemia* . It appears, though, that tubulin synthesis is a minor component compared to the large amount of other proteins

produced. More generally, it is clear that a small tubulin family can support the numerous microtubule activities found in a complex animal, and the tubulin family need not include detyrosinated α-tubulin. These data tend to support the theory that tubulins are multifunctional.

APPENDIX 1

Solutions and Gel Recipes

Unless otherwise noted, all chemicals were reagent grade and were purchased from Sigma Chemical Co. or BDH Inc.

Hatch medium

	g/6L
NaCl	148.2
KCl	4.2
$MgSO_4$	37.6
$MgCl_2 \cdot 6H_2O$	27.7
$CaCl_2$	1.3
$NaHCO_3$	0.24
Na borate	12.0

Pipes buffer

100 mM PIPES
1 mM $MgCl_2 \cdot 6H_2O$
1 mM EGTA

PIPES as free acid was purchased from Research Organics Inc., Cleveland, Oh.

Proteolytic Inhibitor Solutions

Solution A

5.0 mg/mL leupeptin, 1.0 mg/mL trypsin soybean inhibitor in 100 mM Pipes, pH 6.5

Solution B

2 mg/mL PMSF, 1 mg/mL pepstatin in ethanol.

Coomassie blue stain

0.125% Coomassie Brilliant Blue R-250 (Bio-Rad)
50% (v/v) methanol
10% (v/v) acetic acid

SDS-polyacrylamide gel electrophoresis destain

7% acetic acid
20% methanol
5% glycerol

Isoelectric focusing gel

2.75 g urea (Fisher)
0.5 mL 38% acrylamide
1.0 mL 10% NP40
1.25 mL dH_2O

After urea was dissolved, Bio-Lyte ampholytes (Bio-Rad) were added:

200 µL 5/7
50 µL 3/10

The solution was deaerated and polymerized by the addition of 3.5 µL TEMED (BRL Ultra-Pure) and 5 µL 10% ammonium persulfate.

IEF overlay solution

2.75 g urea
200 µL Bio-Lyte 5/7 ampholyte
50 µL Bio-Lyte 3/10 ampholyte
dH_2O to 5 mL.

IEF anode solution

(10 mM) 2.04 mL phosphoric acid in 3 L dH_2O.

IEF cathode solution

(1 mM) 1.55 g histidine in 1 L dH_2O.

IEF gel fixative

4.0% sulfosalicylic acid
12.5% trichloroacetic acid

IEF gel stain

27% (v/v) isopropanol
10% (v/v) acetic acid
0.04% Coomassie Brilliant Blue R-250
0.5% $CuSO_4$, added last to avoid precipitation

IEF gel destain I

12% (v/v) isopropanol
7% (v/v) acetic acid
0.5% $CuSO_4$

IEF gel destain II

7% acetic acid
5% methanol

Two-dimensional PAGE denaturing solution

12.5% (v/v) SDS-PAGE stacking gel buffer
1.5% SDS
10% glycerol
5% mercaptoethanol

1X TAE

40 mM Tris-Acetate
1 mM EDTA

TEMN buffer

0.02 M Tris pH 8.3
0.2 mM $MgCl_2$
0.25 M NaCl
2% Triton X-100
0.95% EDTA

0.1% diethylpyrocarbonate (DEPC) added after the pH is adjusted, and the solution is stirred vigorously for at least 20 min and then autoclaved. Once cooled, 1 mM DTT is added.

TMES buffer

10 mM Tris pH 8.0
5 mM MgCl2
0.5 mM EDTA
0.25 M sucrose (RNAse-free)

Autoclave.

Oligo dT cellulose loading buffer

20 mM Tris pH 7.5
1 mM EDTA
0.5 M NaCl

Autoclave.

0.1% SDS, from a 20% stock solution which is heat-treated at 65 °C for 1 h. Add after the solution has been autoclaved.

Oligo dT cellulose regenerating solution

0.1 M NaOH
5 mM EDTA

Autoclave.

Oligo dT cellulose 2X loading buffer

Made as for loading buffer, but at twice the concentration of all ingredients. Autoclave.

Oligo dT cellulose eluting buffer

20 mM Tris pH 7.5
1 mM EDTA

Autoclave.

0.05% SDS, from a 20% stock which had been heat-treated at 65 °C for 1 h. Add after autoclaving.

TE pH 8.0

10 mM Tris·Cl (pH 8.0)
1 mM EDTA (pH 8.0)

10X RNA gel buffer

0.2 M MOPS (3-(N-morpholino)propanesulfonic acid)
50 mM sodium acetate
10 mM disodium EDTA
pH 7.2, autoclave.

1X RNA electrophoresis buffer

10X RNA gel buffer diluted 1:10
6% formaldehyde

RNA formaldehyde gel loading buffer

50% glycerol
1 mM disodium EDTA
0.4% bromophenol blue
0.4% xylene cyanol

Northern denaturing solution

0.05 M NaOH
1 mM NaCl

Northern neutralizing solution

10 mM Tris pH 7.5

RNA hybridization medium

50% deionized formamide
50 mM sodium phosphate buffer pH 6.5
5X SSC
5X Denhardt's solution
0.1% SDS
100 µg/mL denatured, sheared salmon sperm DNA
10 µg/mL polyriboadenylate

Scintillation fluid

6 g/L 2,5-diphyenlyoxazole (PPO; Fisher)
0.15 g/L 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP; Amersham/Searle)
in toluene.

250 µM taxol

5 µL 250 mM taxol stock, in DMSO
95 µL DMSO
100 µL Pipes buffer, pH 6.2

5X Pipes buffer

0.5 M Pipes
5 mM $MgCl_2$
5 mM disodium EDTA
pH 6.5

15% sucrose cushion

100 mM Pipes buffer pH 6.5
4 M glycerol
15% sucrose

20% sucrose cushion

As above, but 20% sucrose.

Blotting electrode buffer

25 mM Tris
192 mM glycine
20% methanol
pH not adjusted.

TBS-Tween

10 mM Tris pH 7.4
140 mM NaCl
0.1% Tween-20

High Salt Tween (HST)

10 mM Tris pH 7.4
1 M NaCl
0.5% Tween-20

TBS

10 mM Tris pH 7.4
140 mM NaCl

Horseradish peroxidase substrate solution

94 mL TBS
18 mg 4-chloro-1-naphthol
0.025 mL 30% H_2O_2

Alkaline phosphatase buffer

100 mM Tris pH 9.5
100 mM NaCl
5 mM EDTA

Alkaline phosphatase substrate solution

82.5 µL 5-bromo-4-chloro-3-indolyl phosphate (Promega; final concentration 0.21 mg/mL)
165 µL Nitro blue tetrazolium (Promega; final concentration 0.33 mg/mL)
in 5 mL alkaline phosphatase buffer

Alkaline phosphatase stop buffer

20 mM Tris pH 8.0
5 mM EDTA

25 mM TBS

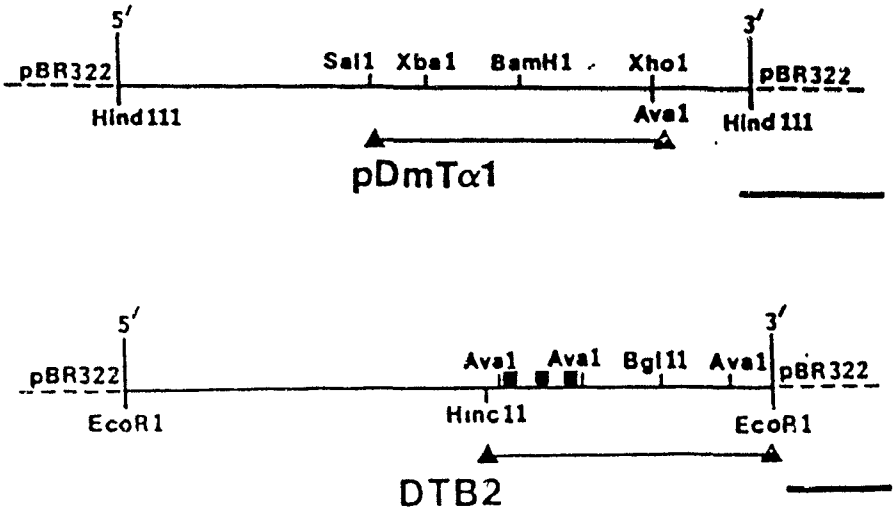
25 mM Tris pH 7.4
140 mM NaCl

APPENDIX 2

Partial restriction maps of plasmids pDmTα1 and DTB2

The *Drosophila* α- and β-tubulin gene clones in plasmids pDmTα1 and DTB2, respectively, were used for the analysis of *Artemia* tubulin mRNA. The limits of the *Drosophila* DNA inserts are designated by vertical lines labelled 5' and 3', while tubulin coding sequences within the insert are indicated by interconnected triangles under the maps. In order to ensure that the use of whole plasmids was yielding accurate results on Northern and dot-blots, specific α- and β-tubulin coding sequence probes were isolated. A segment of the plasmids pDmTα1 containing only α-tubulin coding sequence was obtained by digesting the plasmid with *Bam*HI and *Xho*I and isolating the 900 bp fragment that makes up the 3' end of the coding sequence. A 1500 bp fragment of β-tubulin coding region bounded by the central *Ava*I site and running in the 3' direction to the last *Ava*I site was similarly obtained. The bars indicate 1 kB. Partial restriction maps may be found on the following page.

APPENDIX 2 CONT'D



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