SYNTHESIS AND STORAGE OF MICROTUBULE PROTEINS BY SEA URCHIN EMBRYOS

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ABSTRACT

Studies employing colchicine binding, precipitation with vinblastine sulfate, and acrylamide gel electrophoresis confirm earlier proposals that *Arbacia punctulata* and *Lytechinus pictus* eggs and embryos contain a store of microtubule proteins. Treatment of 150,000 g supernatants from sea urchin homogenates with vinblastine sulfate precipitates about 5% of the total soluble protein, and 75% of the colchicine-binding activity. Electrophoretic examination of the precipitate reveals two very prominent bands. These have migration rates identical to those of the A and B microtubule proteins of cilia. These proteins can be made radioactive at the 16 cell stage and at hatching by pulse labeling with tritiated amino acids. By labeling for 1 hr with leucine-^{3}H in early cleavage, then culturing embryos in the presence of unlabeled leucine, removal of newly synthesized microtubule proteins from the soluble pool can be demonstrated. Incorporation of labeled amino acids into microtubule proteins is not affected by culturing embryos continuously in 20 μg/ml of actinomycin D. Microtubule proteins appear, therefore, to be synthesized on "maternal" messenger RNA. This provides the first protein encoded by stored or "masked" mRNA in sea urchin embryos to be identified.

INTRODUCTION

Sea urchin embryos cultured from fertilization in the presence of sufficient actinomycin D to abolish RNA synthesis cleave, hatch, and grow active cilia (1, 2). This observation suggested to us that mitotic and ciliary microtubule proteins are either stored or are synthesized on stored maternal mRNA ("masked" mRNA) templates.

In early development, sea urchin embryos perform extremely rapid cell divisions, i.e. they progress from one very large cell to approximately 1000 small ones in 5–8 hr, while the mass of the embryo remains constant. Storage of macromolecules and of cytoplasmic organelles by the egg for use in this period, during which they are not being synthesized, is well known: examples are yolk granules, ribosomes, mitochondria, and tRNA. The storage of microtubule proteins has been an oft suggested possibility. Furthermore, it has been shown that sea urchin embryos contain mRNA synthesized during oogenesis, retained by the egg cytoplasm in stable but untranslated form until protein synthesis is stimulated by the process of fertilization (3–5). If RNA synthesis in the embryo is abolished with actinomycin D, these messengers alone direct the protein syntheses necessary for development from the fertilized egg to a normal-appearing hatched blastula.

With respect to microtubules and their mono-

mers, two possibilities are (a) that the monomers are stored and polymerized as needed, with no synthesis taking place during cleavage; and, (b) alternatively, that the monomers are synthesized as needed for structure formation, there being no important storage form. The present report deals with experiments on synthesis and storage of microtubule proteins and of their mRNA templates in the sea urchin embryo. The results suggest that possibilities (a) and (b) are too exclusive, and that, instead, a pool of microtubule monomers *does* exist, and *is* replenished throughout development by synthesis.

MATERIALS AND METHODS

Culture of Embryos and Preparation of Homogenates

Arbacia punctulata were obtained from Mr. Glendle Noble, Panama City, Fl. *Lytechinus pictus* were obtained from Pacific Biomarine Supply Co.,Venice, Calif. Gametes were collected from *Arbacia* by dissection, and from *Lytechinus* by injection of 0.55 M KCl (6). Embryos were cultured as described previously (7) except that culture temperatures were generally 18–20°C.

Embryos were harvested by low speed centrifugation, washed in fresh sea water, then once in a small volume of homogenizing buffer. Homogenization was carried out in two times the packed embryo volume of cold buffer (4°C) containing 0.24 M sucrose, 0.01 M Tris, pH 7.0 (pH adjusted at 25°C) (8).

The homogenate was spun at 27,000 g for 15 min. The resulting supernatant was centrifuged at 150,000 g for 90 min, and the supernatant was retained for isolation of microtubule proteins.

Colchicine-Binding Assay

Colchicine-binding capacity was used for the identification of soluble microtubule protein (9). Assays were performed by the procedure of Borisy and Taylor (10). The 150,000 g supernatant was incubated for 60 min at 37°C with colchicine-^{3}H (final concentration 2.3 $\times$ 10^{-6} M, 2 μCi/ml), then cooled to 0°C. The sample was then run through a 1.25 $\times$ 25 cm Sephadex G-100 column (Pharmacia Fine Chemicals Inc., Uppsala, Sweden) and eluted with 0.01 M pH 7 phosphate buffer at 4°C to separate protein-bound colchicine from unbound colchicine. Protein determinations were made by the method of Lowry et al. (11).

Vinblastine Sulfate Precipitation

Vinblastine sulfate precipitations were carried out as described by Olmsted et al. (8). To the 150,000 g

supernatant there was added 0.1 volume of a 2 $\times$ 10^{-2} M solution of vinblastine sulfate. The sample was mixed and allowed to stand at 0°C for 30 min; the resulting precipitate was collected by centrifugation at 50,000 g for 20 min.

Acrylamide Gel Electrophoresis

Microtubule proteins were examined on 5% acrylamide gels containing 8.0 M urea (6 $\times$ 50 mm, 3 ma/gel, pH 8.3 at room temperature). Gels were prepared as follows: three parts of solution A (24 g urea, 6.05 g Tris [trishydroxyaminomethane] base, 8.0 ml NHCl, 0.09 ml N,N,N',N'-tetramethylethylenediamine [TEMED], 5 ml hexane-extracted glycerol, water to final volume of 50 ml) were added to four parts of solution B (24 g urea, 5 g recrystallized acrylamide, 0.25 g recrystallized methylene (bis) acrylamide [MBA], 5 ml glycerol, water to final volume of 50 ml). The mixture was degassed by connection to a vacuum line for 2 min, and one part of solution C (3 mg/ml ammonium persulfate in 8 M urea) was added. The mixture was then pipetted into acid-washed glass tubes (inner diameter 6 mm) and layered with water containing 1% Triton X-100. Gelling time was about 20 min. No sample or spacer gels were used. Running buffer was Tris glycine adjusted to pH 8.3 (3 g Tris base, 14.4 g glycine, water to 1 liter). Standard running time for electrophoresis was 2.5 hr.

Gels were stained with fast green as described by Gorovsky et al. (12). The color developed is directly proportional to the amount of protein present, and there is no metachromasia. Microtubule proteins were also examined on 5% gels containing sodium dodecyl sulfate (SDS) (13). Samples were prepared for electrophoresis by the method of Renaud et al. (14), except that in most cases here too little sample was available for dialysis and alkylation. Samples were therefore dissolved and reduced in a small volume of 0.35 M, pH 8.8 Tris buffer at 25°C, containing 0.1% ethylenediamine tetraacetate (EDTA) and 8.0 M urea, and made 0.12 M in mercaptoethanol just before use. After about 15 min the sample was layered on the gel. Radioactivity in gels was determined by cutting gels into 1-mm thick slices which were shaken overnight at room temperature in scintillation vials containing 0.4 ml of quaternary amine (NCS) solubilizer (Nuclear-Chicago, Des Plaines, Ill.) and two drops of water. 10 ml of scintillant containing 3.96 g 2,5-diphenyloxazole (PPO) and 0.05 g p-bis[2-(5-phenyloxazolyl)]benzene (POPOP) per liter were added.

Isolation of Cilia and Sperm Tails

Cilia were isolated according to the procedure described by Auclair and Siegel (15) and purified by

centrifugation on 0–60% sucrose gradients at 1000 g for 20 min (15).

Tails from sea urchin spermatozoa were used to provide microtubule protein standards for gel electrophoresis. Sperm tails were prepared by the method of Stephens et al. (16). Microtubule proteins of the outer fibers were prepared by procedure 2 of Renaud et al. (14).

Biochemicals

Actinomycin D was purchased from Mann Research Labs Inc., New York; vinblastine sulfate was a generous gift of Eli Lilly & Co., Indianapolis, Ind. Colchicine-^{3}H-methoxy was obtained from New England Nuclear Corp., Boston, Mass., and mixed-L-amino acids-^{3}H and L-leucine-^{3}H were obtained from New England Nuclear Corp. and International Chemical & Nuclear Corporation, Burbank, Calif.

RESULTS

Measurement of Pool of Colchicine-Binding Proteins

Borisy and Taylor (10) found that there is a considerable amount of colchicine-binding protein in the cytoplasm of sea urchin eggs. This observation was confirmed and extended by determination of the colchicine-binding activity of 150,000 g supernatants from sea urchin eggs and embryos at various stages. Protein-bound colchicine was eluted from a Sephadex G-100 column. Specific activity was calculated as counts per minute of colchicine-^{3}H bound per milligram of protein eluted from the column. Biological variability, and the considerable complexity of the procedure, produced a good deal of scatter in our data (Fig. 1), but a reasonable interpretation of them seems to be that there is a relatively constant level of colchicine-binding activity from fertilization through the gastrula stage.

The embryos used in each of the experiments described in Fig. 1 showed normal and synchronous development, so that the scatter cannot be attributed to abnormal embryos. It will be important, obviously, to obtain many more results before firm conclusions can be drawn as to the constancy or inconstancy of the pool. Important for the present argument, however, is the clear result that a pool exists at all stages studied, and that if its size does fluctuate, the changes do not exceed ±40%.

Since there is very little protein synthesis in the

unfertilized egg (17), the colchicine-binding protein studies suggest that microtubule proteins are stored up during oogenesis for use in embryogenesis, and that a pool of microtubule proteins is maintained in embryogenesis.

Vinblastine Precipitation of Colchicine-Binding Proteins

In order to isolate microtubule proteins, we explored the use of vinblastine sulfate, which precipitates microtubules in vivo (18, 19). It has also been used with notable success in the isolation of a microtubule protein from neuroblastoma cells by Olmsted et al. (8), whose procedure we used. Vinblastine was added to 150,000 g supernatants prepared from whole embryos, and the precipitate
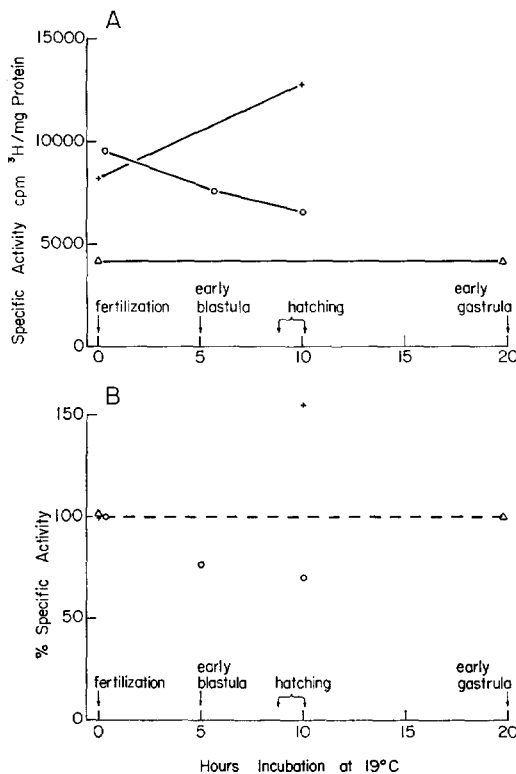

Figure 1 Colchicine binding by A. punctulata proteins during early development. Fig. 1, A: Results of three separate experiments in which the specific colchicine-binding activity was measured just before fertilization and at various stages during development. Fig. 1, B: The results from Fig. 1, A normalized by setting the specific activities of the unfertilized eggs at 100% for each experiment.

was allowed to form at 0°C. Typically, the precipitate contained 2–5% of the protein originally present in the 150,000 g supernatant. Vinblastine precipitated most of the colchicine-binding material from these supernatants, as illustrated by the following experiment.

A 150,000 g supernatant of *A. punctulata* eggs was incubated for 60 min with colchicine-^{3}H as described in Materials and Methods, then treated with vinblastine sulfate. The vinblastine precipitate was dispersed in homogenizing buffer containing 0.1 M guanidine triphosphate (GTP). (GTP and Mg^{++} stabilize colchicine binding, e.g. reference 8). A sample was put onto a Millipore filter (Millipore Corp., Bedford, Mass.), and washed as quickly as possible with several portions of the same cold buffer. Radioassay showed that 75% of the counts bound by the 150,000 g supernate were recovered in the vinblastine precipitate (Table I). There was, furthermore, a large enrichment (fivefold) of the specific activity of colchicine binding. These results agree with those of Olmsted et al. (8) for the precipitation by vinblastine of colchicine-binding protein from neuroblastoma cells.

Vinblastine-Precipitated Proteins and Sperm Tail Microtubules

Protein precipitated by vinblastine was compared by acrylamide gel electrophoresis with known microtubule proteins of sperm tails. Vinblastine-precipitated protein pellets were dissolved in 0.1 ml of 8 M urea buffer containing 0.12 M mercaptoethanol, and samples were layered on urea gels, which were generally run for 2.5 hr and then stained with fast green. One prominent band appeared on these gels. As is shown in Figs. 2 and 3, it corresponds in position to the microtubule protein band of sperm tails. The same result was

TABLE I

Vinblastine Precipitation of Protein-Bound Colchicine from the 150,000 g Supernatant of A. punctulata

Fraction	Total counts bound	SA
	cpm	*cpm/mg protein*
150,000 g supernatant	27,200	1518
Vinblastine ppt	15,210	7605
Vinblastine supernatant	7,030	393

obtained with SDS gels stained with Coomassie blue (results not shown). This band was often resolvable into two bands on urea gels: the resolution obtained seemed related to differences between batches of gels, and was also a function of the amount of protein loaded. For example, the gels shown in Figs. 2 and 3 did not resolve two bands, but a gel run simultaneously, onto which approximately 1/5 as much of the same protein sample was run, did resolve two bands very clearly (e.g., Fig. 6). This band or pair of bands was absent from electrophorograms of vinblastine supernatants (Fig. 4), and, as may be seen by comparing Figs. 2 and 3 with Fig. 4, the vinblastine precipitate represents a highly selective removal of protein from the 150,000 g supernatant.

Selective precipitation of colchicine-binding activity from the 150,000 g supernatant by vinblastine, together with the electrophoretic identity of such precipitated proteins with sperm tail microtubule proteins, argues strongly for the conclusion that colchicine-binding, vinblastine-precipitated proteins in the soluble phase are microtubule proteins. The two protein peaks resolved from our vinblastine precipitates resemble the A and B axonemal proteins of cilia, flagella, and sperm tails which have been studied on urea gels (12, 14, 20). The B tubule protein has a slightly higher electrophoretic mobility under these conditions than the A protein (20). These two are also demonstrable on electrophoresis of whole dissolved *A. punctulata* blastula cilia (Fig. 5). Until, however, we have better characterized the proteins from the two peaks on gels of our vinblastine precipitates, we cannot be sure that *both* peaks contain microtubule proteins. The reason for this caution is the demonstration by Wilson et al. (21) that vinblastine may precipitate efficiently several proteins in addition to the monomers of microtubules. This potential difficulty is probably eliminated by the conditions that we used for vinblastine precipitation. Wilson et al. (21) found that some contaminatory proteins, e.g. from the sea urchin embryo hyaline layer, are precipitated at 37°C *but not* at 0°C. All our treatments with vinblastine were done at 0°C. Furthermore, our 150,000 g supernatants were carefully prepared to avoid the presence in them of ribosomes and other elements of the 150,000 g pellet. Proteins remaining soluble at 150,000 g comprise approximately 30% of the total protein of *L. pictus* embryos. Only 2–5% of this protein was precipitated
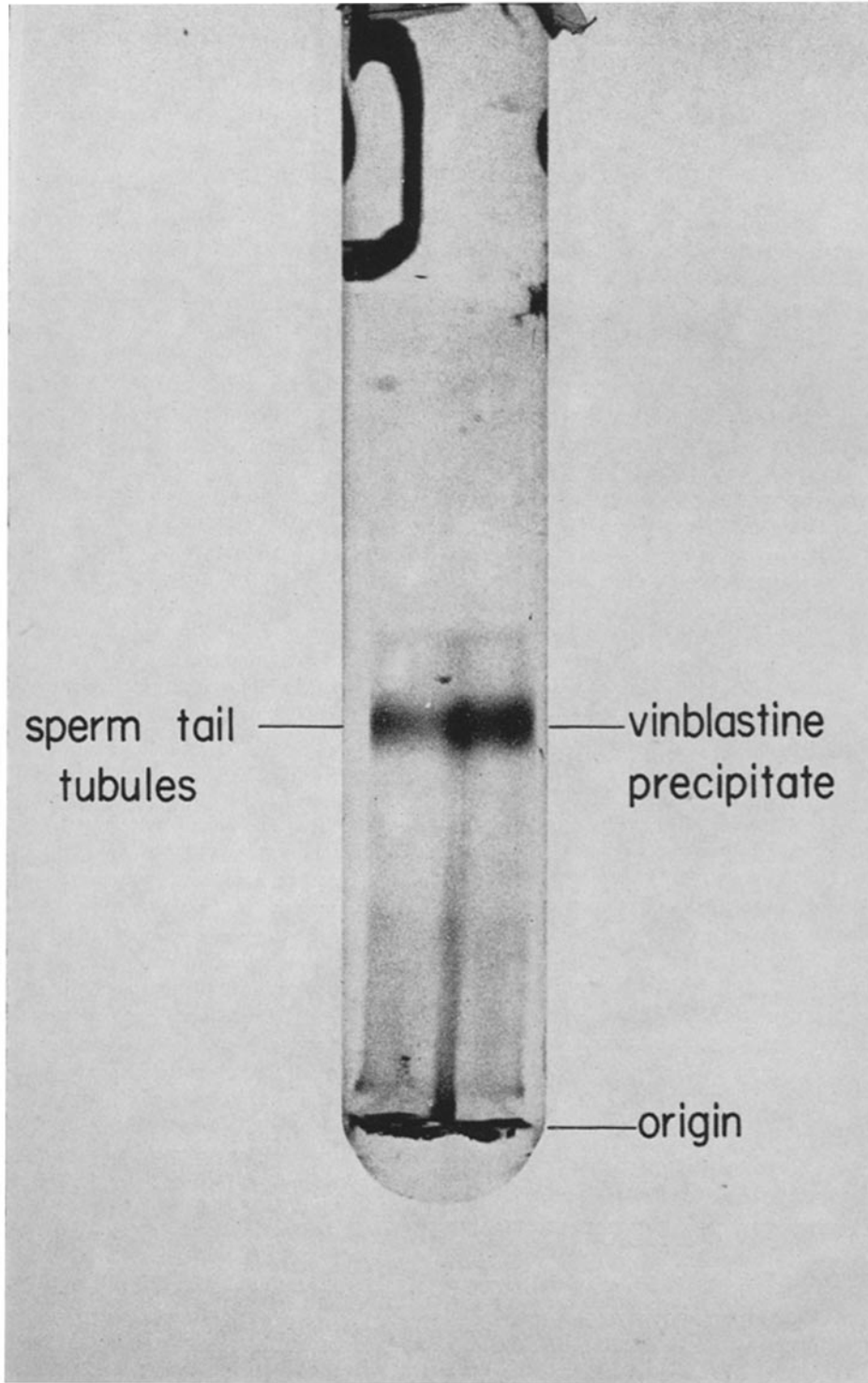
FIGURE 2 A split polyacrylamide gel on one side of which was applied a preparation of solubilized sperm tail protein, while the other side was loaded with a solubilized vinblastine precipitate from the 150,000 g supernatant of *L. pictus*.
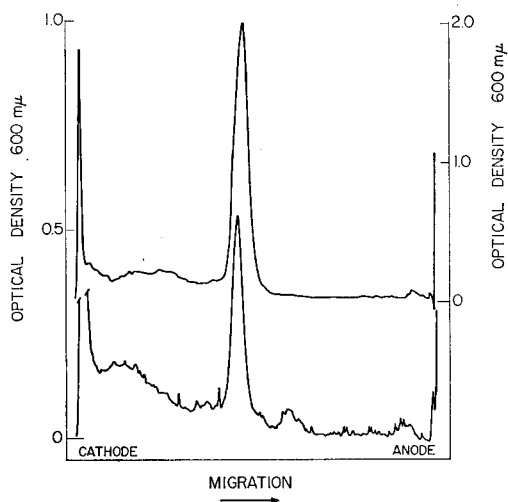
FIGURE 3 Scans of paired gels showing electrophoretic identity of vinblastine-precipitated microtubule proteins from *L. pictus* (upper trace) and microtubule proteins of sperm tails (lower trace).
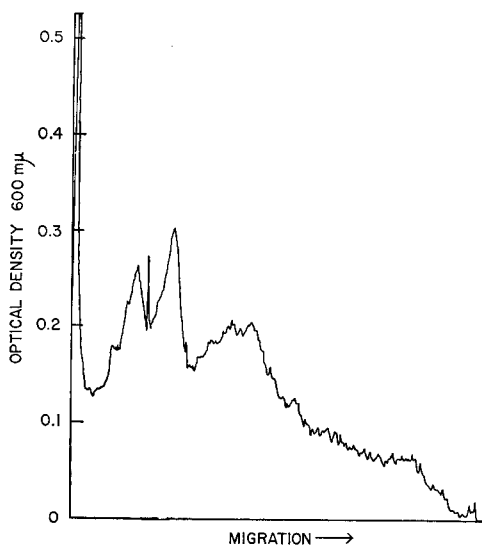

FIGURE 5 Acrylamide gel electrophoresis of solubilized whole *A. punctulata* blastula cilia.

FIGURE 4 Acrylamide gel electrophoresis of sea urchin embryo proteins remaining in a 150,000 *g* supernatant from which the vinblastine-precipitatable proteins have been removed.

by vinblastine. The electrophoretic gels show that in our system the precipitation by vinblastine was highly selective, and that the presumptive microtubule proteins thus precipitated migrated electrophoretically like sperm tail tubule proteins in both urea and SDS gels. Thus, we feel that we
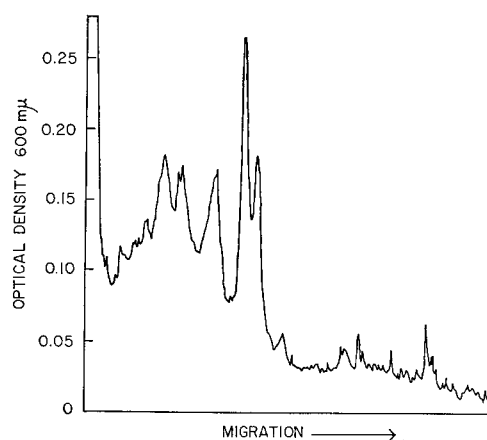
avoided wholesale, nonspecific precipitation such as occurred in the experiments of Wilson et al. (21) on chick brain proteins.

Synthesis of Microtubule Proteins

Despite the fact that microtubule-containing organelles such as cilia are produced at certain stages of early development, the pool of soluble microtubule proteins seems to remain relatively unchanged. This suggested the interrelated possibilities of synthesis and turnover of microtubule proteins in maintenance of the pool.

Synthesis could be demonstrated by labeling *L. pictus* embryos for 30 min with mixed amino acids–³H, followed by preparation of vinblastine precipitates which were then examined on gels (Fig. 6). A culture of *L. pictus* embryos (2 ml of packed embryos/100 ml) was split into two 100-ml cultures. When the 16 cell stage was reached, the first culture was labeled with 1 μCi/ml of mixed amino acids–³H (NEN mixture G) for 30 min. The second culture was labeled in the same way at hatching. Both microtubule protein peaks were labeled in early cleavage (16 cell stage) as well as in hatching blastulae.

Since the soluble microtubule proteins could be labeled, we investigated the incorporation of labeled amino acids into the microtubules proper of cilia, when they appear at hatching. Ciliary microtubules were in fact labeled in embryos exposed to leucine–³H at hatching (Fig. 7) and at gastrula (Fig. 8). At hatching, soluble pool microtubule proteins as well as ciliary microtubules in-
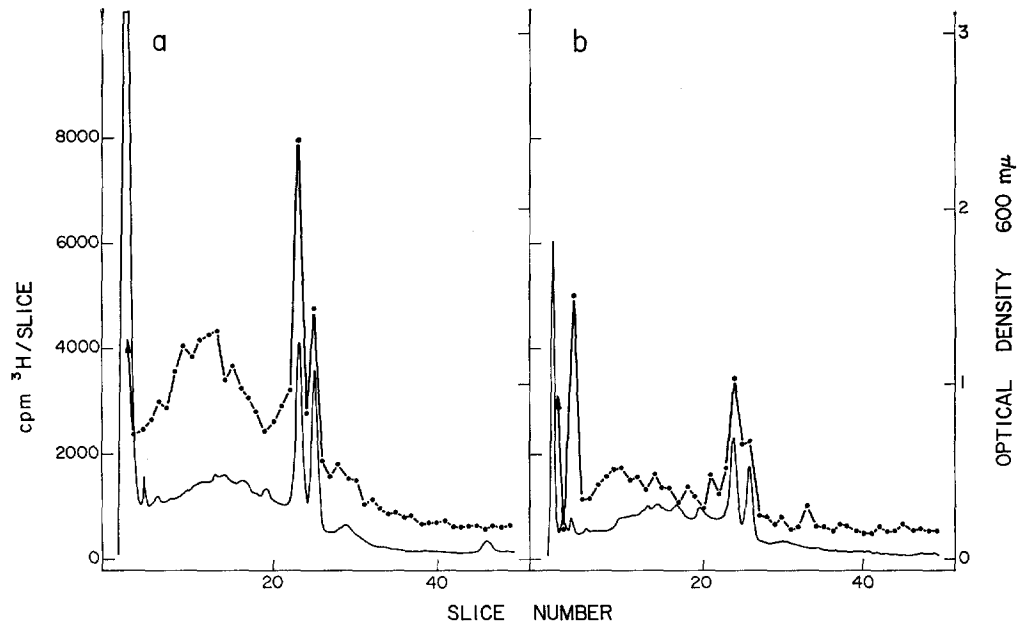
FIGURE 6 Synthesis of microtubule proteins by embryos of *L. pictus*. Fig. 6 *a*: Cleavage stage embryos (16 cells). Fig. *b*: Hatching blastula stage embryos. Embryos were incubated for 60 min in the presence of mixed amino acids-^{3}H at the stages indicated, and then used for the preparation of the vinblastine precipitates which were run on polyacrylamide gels. The prominent OD peaks (continuous line) are bulk microtubule proteins. The fast-running peak (right) corresponds to the B tubulins of ciliary axonemes, and the slower running peak to the A tubulins. ^{3}H counts per slice are indicated by • - •.
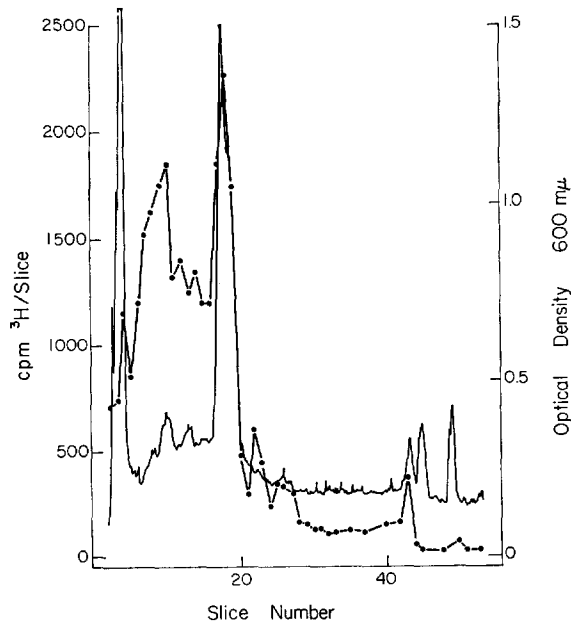

FIGURE 7 Labeling of whole ciliary proteins by hatching blastulae of *A. punctulata*. Embryos were incubated with leucine-^{3}H for 90 min, harvested, and the cilia were prepared. The ciliary pellet was dissolved and reduced, as described in Materials and Methods, and run on a urea gel. The tall OD peak is that of microtubule proteins. Continuous tracing indicates OD, • - • indicates ^{3}H counts per slice.
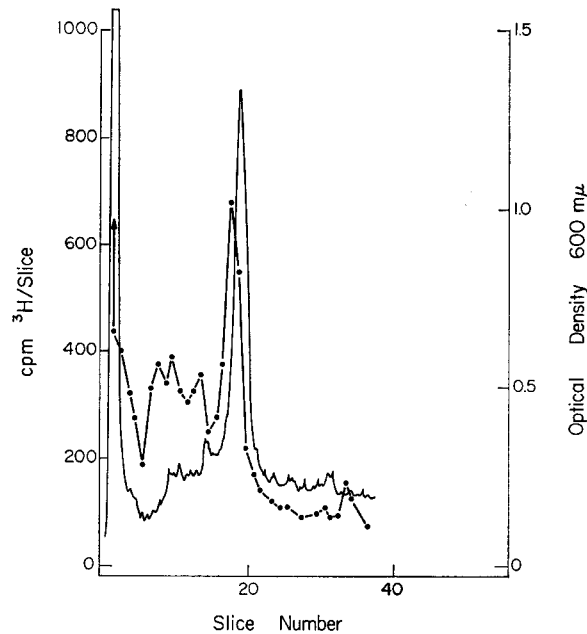
FIGURE 8 Labeling of whole ciliary proteins by gastrulae of *A. punctulata*. The experiment is as described in the legend for Fig. 7, except that gastrulae were incubated with leucine-^{3}H for 60 min.

TABLE II

Specific Activities of Leucine-^{3}H Incorporation into Soluble Microtubule Proteins of L. pictus

Sample	Total counts under microtubule protein peaks	SA*
	cpm	
End of "pulse"	10,171	293 (1.00)
End of "chase"	6732	167 (0.57)

* Specific activity equals counts per minute per milligram of paper weight (region of densitometer trace under microtubule protein peaks, cut out and weighed). Values in parentheses are relative specific activities.

corporate labeled amino acids. It is of interest to note that the specific activity of the ciliary matrix proteins is higher than that of the microtubules. A similar differential of specific activity was observed by Gorovsky et al. (12) in regenerating and non-regenerating *Chlamydomonas* flagella. This result they interpreted as being due to a more rapid turnover of matrix proteins than microtubule proteins, an effect that could in turn be due to the existence of a larger pool of soluble ciliary microtubule proteins than of matrix proteins. There is certainly a pool of flagellar proteins in *Chlamydomonas* (22), and we suggest that similarly

a pool of ciliary proteins, including microtubule monomers, exists in urchin embryos.

Turnover of Soluble Microtubule Proteins

The question of turnover of soluble microtubule proteins was approached by labeling a culture containing 4 ml of packed embryos in 200 ml of sea water with 200 μCi of leucine-^{3}H (L-leucine 4,5-^{3}H, 29.8 Ci/mmole) for 60 min commencing at the 16 cell stage. At the end of the labeling period, half of the culture was used for immediate precipitation with vinblastine. The remainder of the culture was washed twice with sea water, then resuspended in sea water containing 1 mg/ml of unlabeled leucine. Embryos were cultured for 3 hr longer in the presence of unlabeled leucine, by which time the embryos were unhatched blastulae. These were harvested. The effectiveness of such a leucine chase in embryos was demonstrated by Fry and Gross (7). The results are presented in Table II. During the chase period there was a 43% decrease in the specific activity of labeled soluble microtubule proteins. This indicates that both synthesis and withdrawal are involved in the maintenance of the microtubule protein pool. Microtubule protein synthesis is probably continuous throughout cleavage, although we have not yet determined the time of
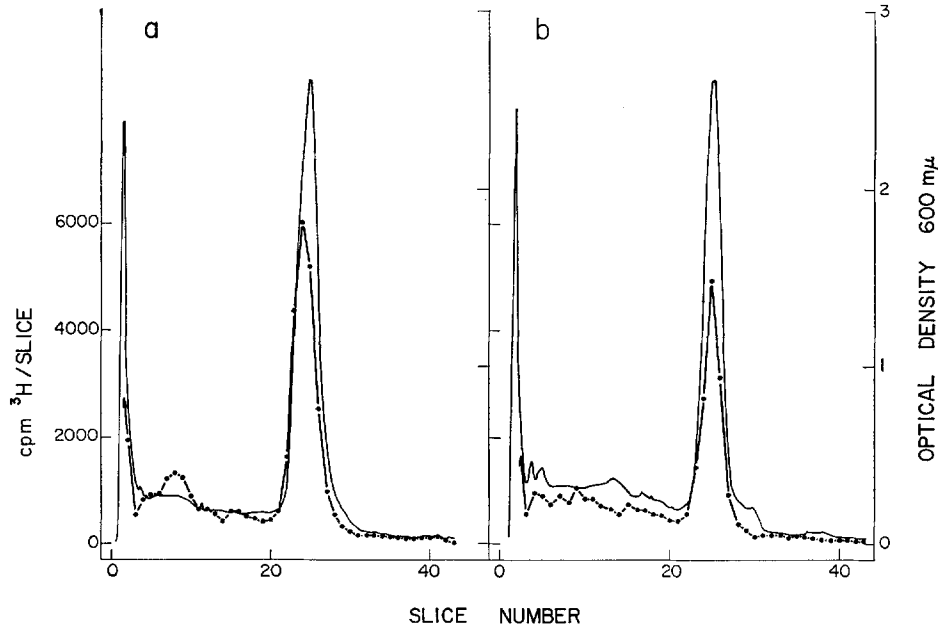
FIGURE 9 Labeling of microtubule proteins of *L. pictus* embryos in the presence and absence of high molecular weight RNA synthesis. Microtubule proteins were precipitated with vinblastine and prepared for gels as previously described. Solid trace indicates OD; ●-● indicates ³H counts per slice. Fig. 9 *a*: Control embryos labeled at 16 cell stage with leucine-³H. Fig. 9 *b*: Embryos cultured in the presence of 20 μg/ml of actinomycin D continuously from 60 min before fertilization. Embryos were labeled at the 16 cell stage the same as controls.

its inception. The results of Mangan et al. (23, 24) suggest that synthesis of mitotic microtubule proteins begins before first cleavage.

Effect of Actinomycin D on Microtubule Protein Synthesis

In order to select between new or "embryonic" mRNA and maternal mRNA as template for the microtubule protein synthesis, eggs (2 ml of packed eggs per 100 ml of sea water) of *L. pictus* were incubated continuously with 20 μg/ml actinomycin D from 60 min before fertilization. This dose of actinomycin enters the cells and stops all labeling of RNA in *L. pictus* cleavage-stage embryos, except for that of pCpCpA terminals in tRNA (25, 26). Entry of actinomycin D-³H into both eggs and embryos was demonstrated by radioautography. Inhibition of mRNA synthesis in cleavage stage embryos, already proven in the earlier literature (1), was determined again for our present purpose by preparation of labeled RNA in the presence and absence of actinomycin, and by the analysis of such RNA on sucrose gradients. Actinomycin D (20 μg/ml) was found to inhibit RNA synthesis by over 93% within 90

min of addition of the drug (26). The rate of cleavage is slowed, but the embryos develop nevertheless to normal-appearing blastulae that grow cilia, hatch, and swim.

Embryos cultured under these conditions were labeled with leucine-³H(2 μCi/ml, SA 29.8 Ci/mmole) for 60 min starting at the 16 cell stage, and at the end of that time were prepared for study of microtubule proteins. The results of one such experiment are shown in Fig. 9. There was only a slight inhibition of microtubule protein synthesis by actinomycin D, and even this was variable and nonselective. Therefore, all or most of the microtubule proteins are synthesized on maternal mRNA, since the blocking of new mRNA production has a negligible effect.

DISCUSSION

Along with several other classes of macromolecules and organelles stored in sea urchin eggs for use in embryogenesis, microtubule proteins and the mRNA species required for their continued synthesis are laid down during oogenesis. A pool of soluble microtubule proteins was found to be present from egg to gastrula, as measured by

colchicine binding, and the size of the pool did not vary greatly. These proteins were precipitable and partially purified by the use of vinblastine sulfate.

Two prominent bands were identified in electrophoretic gels of vinblastine precipitates. These bands on gels migrate like the A and B axoneme proteins of cilia and flagella. At present, there is no *direct* evidence that these soluble proteins serve as ciliary microtubule precursors, but ciliary microtubules do incorporate labeled amino acids when labeling is done at hatching or at the gastrula stage.

Labeling experiments indicated that both species of presumptive soluble microtubule proteins in the embryos are synthesized at early cleavage as well as at the hatching stage. Since a constant pool seems to be maintained, it is likely that the pool is turning over. A 1-hr pulse with leucine-^{3}H followed by 3 hr of culture in the presence of unlabeled leucine resulted in a 43% "chase-out" of the label from the *soluble* microtubule protein pool, indicating a rapid outflow via either degradation or assembly into structures.

A hypothetical general scheme for such pool turnover in sea urchin embryos can be diagrammed as follows. It must be stressed that, although only one pool is indicated, there are at least four species of microtubules known (27). It has not yet been established how many species of microtubule proteins actually exist, but there may well be at least two (8, 20), and each may have its own pool, not necessarily comparable in size nor in utilization to the others.
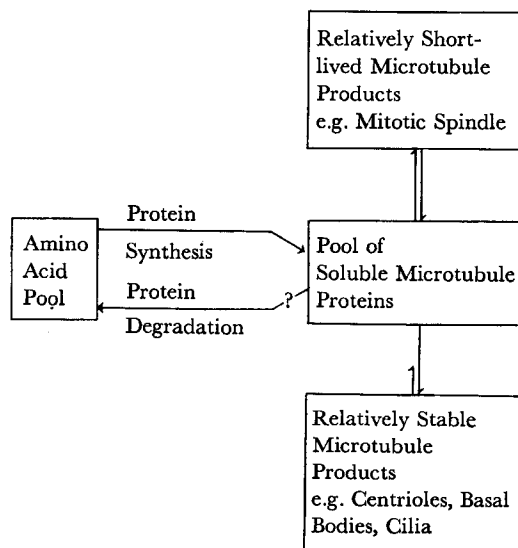
The evidence obtained in these experiments indicates a contribution from protein synthesis during development to a microtubule protein pool, which is also turning over. We also know from the work of Mangan et al. (23) that spindle fibers incorporate labeled amino acids in cleavage stage embryos. We do not know the relative contributions of the processes removing microtubule proteins from the pool, but Fry and Gross (7) showed that there is no detectable degradation of newly synthesized proteins in early sea urchin embryos. The vinblastine-precipitable microtubule proteins make up less than 2–5% of the soluble proteins, however, and undoubtedly a very small fraction of the newly synthesized protein. It is therefore not certain that turnover by degradation of these new proteins could have been detected in pulse-chase studies. It has been argued that the mitotic apparatus functions by a polymerization-depolymerization mechanism (28–30) which requires a soluble pool of microtubule subunits. The amount of microtubule protein required for assembly of the first cleavage mitotic apparatus may be rather large, since Cohen and Rebhun (31) have calculated that at least 0.1% of the protein of the embryo in first metaphase is microtubule protein in spindle fibers. They argue that most of this protein is present in the egg and is not synthesized between fertilization and first cleavage (31), although some of it is synthesized in that period (24).

We have no direct evidence for the existence of a pool in equilibrium with cytoplasmic, nonmitotic microtubules in sea urchin embryos, but neuroblastoma cells, which have a high density of microtubules in the cytoplasm, have a large soluble pool of one species of microtubule protein. Behnke and Forer (27) argue that intracellular microtubules and mitotic microtubules are very similar and may in fact be assembled from the same protein.

The question of a pool of ciliary microtubule proteins is still, in principle, open. Because of the stability of cilia, a large pool of microtubule subunits might not be necessary for assembly of cilia. In organisms in which careful studies relevant to ciliary microtubule protein pools have been made, they have been found to vary widely in size. For example, *Tetrahymena* and several flagellates have no measurable pools (32, 33), while *Chlamydomonas* has a large pool (22). The data reported here are consistent with the existence of a pool of ciliary microtubule proteins. There

have been reported some direct investigations on pools of ciliary proteins in sea urchin embryos (15, 34). These studies, based on the effects of protein synthesis inhibitors on ciliary regeneration, are, unfortunately, contradictory and somewhat inconclusive. Pulse-chase experiments, similar to those described above, are in progress to determine directly the extent to which microtubule proteins synthesized at one stage of embryogenesis are utilized in the assembly of cilia at another stage.

Auclair and Siegel (15) did demonstrate that sea urchin gastrulae experimentally deprived of cilia could regenerate them in the presence of high doses of actinomycin D, and that the incorporation of labeled amino acids into cilia was not impaired, although over-all protein synthesis in the embryo was. However, ciliary proteins were not fractionated: incorporation into whole cilia was measured, so that effects on microtubule proteins in particular are difficult to assess.

Of particular interest for us was the demonstration that microtubule proteins are synthesized mainly or entirely on maternal mRNA. Specific protein products of stable templates have been studied in several differentiating systems: examples are uridine diphospoglucose (UDPG)–pyrophosphorylase and uridine diphosphate (UDP)–galactose polysaccharide transferase from Dictyostelium (35), cocoonase from silk worm pupae (36), lens crystallins (37), and hemoglobin (38, 39). No specific protein has heretofore been identified as a product of maternal templates in sea urchin embryos. It is not known how the masking of the mRNA is accomplished, nor how the mRNA is unmasked to allow its initiation and translation.

It has been difficult, without a specific product protein of maternal mRNA, to select among the many possible mechanisms of masking, for these cover the entire range from sequestration within a protective capsule of protein to presence of a nucleotide sequence that prevents translation. A specific protein product, and the chance for isolation of the corresponding maternal mRNA, may now permit some headway with the problem.

Note Added in Proof: An important supporting datum for Scheme A (relating soluble microtuble proteins to stable and unstable organelles) is provided by the recent demonstration of Bibring and Baxandall (1971. J. Cell Biol. 48:324.) that sea urchin mitotic apparatus contains proteins that are probably identical with the outer doublet proteins of sperm tails. We have now found, in addition (Raff, Colot, and Gross, unpublished data), that microtubule protein synthesis begins before the first cleavage metaphase. Extraction from the mitotic apparatus of microtubule proteins that appear to be identical with those from cilia and from a soluble pool, coupled with the (now) certainty of their continuous synthesis from the beginning of development, raise considerably the level of likelihood for the proposed relations among these compartments.

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