Distribution of Fluorescently Labeled Tubulin Injected into Sand Dollar Eggs from Fertilization through Cleavage

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ABSTRACT Porcine brain tubulin labeled with fluorescein isothiocyanate (FITC) was able to polymerize by itself and co-polymerize with tubulin purified from starfish sperm flagella. When we injected the FITC-labeled tubulin into unfertilized eggs of the sand dollar, *Clypeaster japonicus*, and the eggs were then fertilized, the labeled tubulin was incorporated into the sperm aster. When injected into fertilized eggs at streak stage, the tubulin was quickly incorporated into each central region of growing asters. It was clearly visualized that the labeled tubulin, upon reaching metaphase, accumulated in the mitotic apparatus and later disappeared over the cytoplasm during interphase. The accumulation of the fluorescence in the mitotic apparatus was observed repeatedly at successive cleavage. After lysis of the fertilized eggs with a microtubule-stabilizing solution, fluorescent fibrous structures around the nucleus and those of the sperm aster and the mitotic apparatus were preserved and coincided with the fibrous structures observed by polarization and differential interference microscopy. We found the FITC-labeled tubulin to be incorporated into the entire mitotic apparatus within 20–30 s when injected into the eggs at metaphase or anaphase. This rapid incorporation of the labeled tubulin into the mitotic apparatus suggests that the equilibrium between mitotic microtubules and tubulin is attained very rapidly in the living eggs. Axonemal tubulin purified from starfish sperm flagella and labeled with FITC was also incorporated into microtubular structures in the same fashion as the FITC-labeled brain tubulin. These results suggest that even FITC-labeled heterogeneous tubulins undergo spatial and stage-specific regulation of assembly–disassembly in the same manner as does sand dollar egg tubulin.

Microtubules (MTs)[1] play important roles in various cellular events during early embryonic development, such as pronuclear migration, chromosome movement, and spindle elongation (25, 27, 32, 37). Mitotic MTs that are maintained in an equilibrium with the cellular tubulin pool exhibit pronounced changes in the distribution in the mitotic spindle in living cells, as determined by polarization microscopy (11, 13, 14). The distribution of MTs was also investigated precisely by the electron microscopy and by immunocytochemistry (1, 7, 26). It is, however, unknown how polymerization and depolymerization of tubulin are regulated in living cells.

Tubulin is a conservative protein, as suggested by co-polymerization of a wide range of tubulin species as well as by primary structural and immunochemical analyses, irrespective of the fact that some characteristics of assembly–disassembly of echinoderm egg tubulin are different from those of brain tubulin (17, 18, 21, 28). The isolated mitotic apparatus can incorporate brain MT proteins to construct birefringent structures (12, 31, 33). In addition, birefringence of the mitotic apparatus increases by injection of the heterogeneous tubulin (12). Therefore, it is expected that brain tubulin can be used to construct MT-containing structures in mitotic cells. Because brain tubulin can be obtained in a large quantity, it would be an important tool in investigating the role of MTs in cell motility in vivo.

[1] *Abbreviations used in this paper:* DMSO, dimethylsulfoxide; D/P, molar ratio of dye to protein; FITC, fluorescein isothiocyanate; MAPs, microtubule-associated proteins; MMK, 10 mM 2-(*N*-morpholino)ethanesulfonic acid, 0.5 mM $MgCl_2$, 50 mM KCl (pH 6.8); MT, microtubule; PMEG, 100 mM PIPES or 100 mM MES, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.5 mM GTP (pH 6.8).

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Recently, molecular cytochemistry (41) has become a powerful tool in investigating dynamic roles of physiologically active proteins in living cells (5, 6; for review, also see reference 19). In this method, these proteins or the antibodies against them are labeled fluorescently and introduced into living cells. The behavior of such proteins within the cells and the functions of the cells introduced with them are investigated simultaneously by fluorescence and various light microscopy. More recently, it has been reported that fluorescently labeled brain tubulin is incorporated into fibrous structures when injected into living cells (16, 35, 36, 42).

In the present study, tubulin prepared from porcine brain and outer fibers of starfish sperm flagella was labeled with fluorescein isothiocyanate (FITC). We injected the labeled tubulin into living sand dollar eggs in order to investigate dynamics of MT assembly–disassembly during fertilization and cleavage. From our observation of the injected eggs by use of fluorescence, polarization, and differential interference microscopy we found that the FITC-labeled tubulin was used to construct microtubular structures in the eggs during fertilization and cleavage. A pronounced finding was that the incorporation of the labeled tubulin into those structures occurred very rapidly.

MATERIALS AND METHODS

Preparation of MT Proteins and Tubulin: We prepared MT proteins from porcine brains by two cycles of temperature-dependent polymerization and depolymerization by the method of Shelanski et al. (38) with some modifications. The reassembly buffer solution (PMEG) consisted of 100 mM piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) or 100 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.8), 0.5 mM $MgCl_2$, 1 mM ethyleneglycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), and 0.5 mM GTP. Tubulin was purified from the MT protein fraction by phosphocellulose column chromatography after Weingarten et al. (43) with a minor modification.

We purified outer fiber tubulin from sperm axonemes of the starfish, *Asterias amurensis*. The starfish sperm axonemes were solubilized with a sonifier (Branson cell disrupter 200; Branson Sonic Power Co., Danbury, CT) and centrifuged according to Kuriyama (20). The resulting supernatant was processed by purification of the outer fiber tubulin by one cycle of temperature-dependent polymerization and depolymerization (20, 29). These proteins were stored at −80°C until use.

Preparation of FITC-labeled Proteins: The porcine brain tubulin was labeled with FITC by the two methods described below.

(*a*) After Keith et al. (16), 2 ml of MT proteins (~20 mg/ml) was mixed with an equal volume of PMEG containing 8 M glycerol and incubated at 37°C for 30 min to form MTs. This was mixed with 20 μl dimethylsulfoxide (DMSO) containing 100 mM FITC and incubated at 37°C for 10 min more. The mixture was then centrifuged at 100,000 *g* for 30 min to obtain MT pellets. After being washed with warm PMEG without GTP containing 4 M glycerol, the pellets were homogenized with cold 20 mM PIPES containing 0.5 mM $MgCl_2$, 1 mM EGTA, and 0.5 mM GTP. The homogenate was centrifuged at 100,000 *g* for 30 min at 0°C, and the labeled tubulin was separated by phosphocellulose column chromatography. The flow-through fractions were collected and their solute constituents were adjusted to PMEG containing 4 M glycerol and 5 instead of 0.5 mM $MgCl_2$. This labeled tubulin solution was purified by one more cycle of temperature-dependent polymerization and depolymerization, and was applied to Bio Gel P-10 column to remove free dye and glycerol. The void fractions were collected and stored at −80°C.

(*b*) Phosphocellulose-purified tubulin was assembled into MTs by incubation at 37°C for 30 min in PMEG containing 4 M glycerol and 5 instead of 0.5 mM $MgCl_2$. 5 mM FITC dissolved in DMSO was then added to the assembled MT suspension in a 10:1 molar ratio of FITC to tubulin dimer, and immediately centrifuged at 100,000 *g* for 30 min at 30°C. The labeled MT pellets were homogenized with cold PMEG and centrifuged at 100,000 *g* for 30 min at 30°C. The supernatant was applied to a Bio Gel P-10 column (Bio-Rad Laboratories, Richmond, CA) and the void fractions were stored at −80°C.

In some cases, we used MT proteins labeled with FITC for injection. MT proteins purified by one cycle of polymerization and depolymerization without use of glycerol were assembled into MTs at 37°C for 30 min in the absence of glycerol. The assembled MTs then were combined with FITC solution and further incubated at 37°C for 10 min. After centrifugation, MT pellets were homogenized with cold 1 M glutamate solution containing 0.5 mM GTP and 0.5 mM $MgCl_2$ (pH 6.8) and centrifuged. The supernatant was stored at −80°C until use.

We also labeled starfish sperm flagella outer fiber tubulin with FITC according to the second method described above. Outer fiber tubulin was assembled into MTs in 100 mM PIPES (pH 6.8) containing 1 mM EGTA, 120 mM KCl, 1.5 mM GTP, 3 mM $MgCl_2$, 1 mM dithiothreitol, and 4 M glycerol at 37°C for 30 min. After addition of 100 mM FITC, the mixture was immediately centrifuged at 100,000 *g* for 30 min at 30°C. We washed the precipitates and homogenized them at 0°C with 50 mM PIPES (pH 6.8) containing 0.1 mM $MgCl_2$, 0.2 mM EGTA and 0.5 mM GTP to disassemble labeled MTs. After centrifugation, we subjected the supernatants to gel filtration column chromatography using Bio-Gel P-10 to remove free dye. The void fractions were stored at −80°C.

FITC-labeled denatured tubulin was prepared as follows. The phosphocellulose-purified tubulin was diluted to ~0.5 mg/ml with 10 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.8) containing 0.5 mM $MgCl_2$ and 50 mM KCl, MMK, and followed by incubation at 42°C for 1 h. The tubulin solution was concentrated with Aquacide II-A (Calbiochem-Behring Corp., LaJolla, CA) at room temperature and centrifuged at 30,000 *g* for 10 min. The denatured tubulin in the supernatant was reacted with FITC at a molar ratio of 1:10, incubated for 10 min at 0°C, and gel filtrated. The D/P ratio (molar ratio of dye to protein) of this sample was ~2.5.

We prepared FITC-labeled bovine serum albumin (BSA) by mixing a BSA solution with DMSO containing FITC and then gel filtrating it.

Determination of the Degree of Labeling (D/P ratio): From three independent measurements, the extinction coefficient at 494 nm of FITC in MMK was determined to be 5.45×10^4 by calibration from that (7.54×10^3) in 0.01 M acetate buffer (9). Next, a tubulin solution (200 μl) at a concentration of ~2.2 mg/ml in PMEG was mixed with 10 μl of DMSO containing 5 mM FITC and incubated at 0°C for 4 h. The mixture was then applied to a Bio Gel P-10 column to separate free dye from the dye conjugated with tubulin. We calculated the amount of the conjugated dye by subtracting the amount of the free dye from the original amount of the added dye. This resulted in a value of 2.52×10^4 as the molar extinction coefficient of FITC-labeled tubulin. We obtained the D/P ratio directly by measuring the absorbance at 494 nm of the FITC-labeled tubulin solution and the protein concentration. We determined the concentration of proteins was by the method of Lowry et al. (23) using BSA as a standard.

Polyacrylamide gel electrophoresis was carried out in the presence of SDS according to the method of Laemmli (22).

Polymerization Assay: Polymerization of FITC-labeled porcine brain tubulin or co-polymerization of the labeled tubulin with outer fiber tubulin was followed by measurement of the change in absorbance of the solution (4, 15) at 350 nm with a Gilford 260 spectrophotometer (Gilford Instrument Laboratories, Inc., Corning Glass Works, Oberlin, OH). We initiated the polymerization by transferring tubulin solutions at 0°C into a cuvette prewarmed to 37°C.

The ability of FITC-labeled porcine brain tubulin to co-polymerize with outer fiber tubulin was measured by centrifugation assay as follows. The FITC-labeled porcine brain tubulin was mixed with outer fiber tubulin at various ratios, incubated at 37°C for 20 min, and centrifuged at 30,000 *g* for 30 min to sediment MTs. The MT pellets were homogenized with the same assembly buffer solution (90 mM PIPES [pH 6.8], 80 mM KCl, 0.5 mM $MgCl_2$, 0.4 mM EGTA, and 0.4 mM GTP). Then the homogenate and the above supernatant were diluted with MMK, and dye and protein concentrations were measured.

We prepared flagellar axonemes from the sperm of the sea urchin, *Anthocidaris crassispina*, by the method reported by Stephens (39). Before their use as seeds, we fragmented the axonemes by shearing them 50 times through an injection needle (26 gauge and 0.5 in).

Preparation of Eggs: We obtained gametes of the sand dollar, *Clypeaster japonicus*, by the injection of 0.5 M KCl or sea water containing 1 mM acetylcholine into the coelomic cavity. The fertilization membrane and hyaline layer were removed by treatment of the eggs with 1 M urea for 1 min shortly after insemination.

Microinjection: We carried out microinjection at 25 ± 1°C as described by Hiramoto (10) using a braking micropipette. Fluorocarbon oil (FC-42; Sumitomo-3M Ltd., Tokyo) was used as a brake oil. The volume microinjected into the eggs was usually 1–3% of the egg volume. FITC-labeled brain tubulin at a concentration of 5–11 mg/ml was used for injection, and the labeled tubulin prepared by the second method was mainly used, because both the D/P ratio and the protein concentration of this labeled tubulin were higher than those obtained by the first method. The concentrations of FITC-labeled flagellar outer fiber tubulin and of brain MT proteins were 4 mg/ml. The concentrations of FITC-labeled denatured brain tubulin and FITC-labeled BSA

were 5 and 15 mg/ml, respectively. The tubulin solutions were split into 6–8-μl aliquots and stored at −80°C. Before experimentation, the frozen solutions were thawed and kept on ice. The solutions of FITC-labeled flagellar tubulin were centrifuged at 15,000 rpm for 20 min at 0°C before injection in order to remove aggregates.

Lysis of Injected Eggs: The unfertilized eggs injected with FITC-labeled tubulin were re-placed into Ca-free sea water from normal sea water and then fertilized (the eggs of *Clypeaster japonicus* can be fertilized in Ca-free sea water). The fertilized eggs injected with FITC-labeled tubulin were transferred to Ca-free sea water containing 1 mM EGTA (pH 7.0). We then lysed these eggs by squirting a lysis buffer solution consisting of 10 mM EGTA, 2 mM $MgCl_2$, 150 mM PIPES (pH 7.0), 10% DMSO, and 1% Triton X-100.

Microscopy: We made our observations with a Nikon OPTIPHOT microscope (Nikon, Tokyo) equipped with epifluorescence, polarization (a Brace-Köhler compensator with a polarizer), and differential interference (NT) optics using a UVF 40× (NA 1.30) glycerol immersion lens. Paraffin oil (~1.478 of refractive index) was used as an immersion liquid instead of glycerol. With this microscope, we carried out fluorescence polarization, and differential interference observations within a few tens of seconds without changing the objective lens. We performed fluorescence or polarization and differential interference observations by choosing either the epi-illumination or the transmitting light illumination, respectively, using two shutters put alternately in these light paths. We carried out differential interference or polarization observations by either putting Nomarski prisms in the transmitting light path or removing them. Were recorded images either directly on Kodak Tri-X film using a microscope camera (UFX; Nikon) for which we determined the exposure automatically by setting the ASA to be 800–1600, or on Kodak Plus-X film using a 35-mm camera (XG-E, Minolta Camera Co. Ltd., Tokyo) at an exposure of 0.5 s by photographing a TV monitor (PM-121T; Ikegami Tsushinki Co., Tokyo) coupled to a video camera equipped with a silicon-intensified target tube (C-1000-12; Hamamatsu Photonics, Hamamatsu, Japan). A series of differential interference, fluorescence, and polarization micrographs were taken within a period of 10–30 s. This period depended on the exposure time for fluorescence and polarization micrographs. Unless otherwise stated, the micrographs shown in the present study were taken directly on 35-mm film. The micrographs were sometimes taken with the video camera.

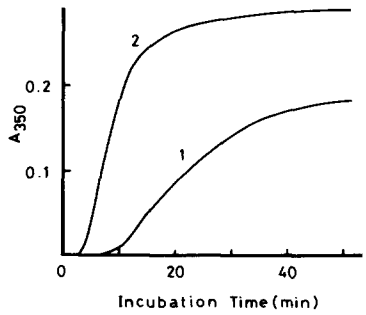

FIGURE 1 Time courses of polymerization of phosphocellulose-purified FITC-labeled (*1*) and unlabeled (*2*) tubulin at 37°C. The same concentration of tubulin (1.2 mg/ml) was polymerized in a buffer solution containing 4 M glycerol, 100 mM PIPES (pH 6.8), 5 mM $MgCl_2$, 1 mM EGTA, and 0.5 mM GTP.

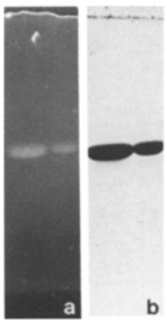

FIGURE 2 SDS polyacrylamide gel electrophoresis of FITC-labeled brain tubulin. (*a*) Fluorescence pattern. (*b*) Coomassie Brilliant Blue staining pattern. 10 (*left lane*) and 3 (*right lane*) μg of FITC-labeled tubulin were electrophoresed.

RESULTS

Fluorescein Labeling of Tubulin

When MTs were first formed from brain MT proteins and then reacted with FITC (see Materials and Methods), FITC was conjugated to both tubulin and microtubule-associated proteins (MAPs). After purification of the tubulin, the D/P ratio was <0.1. This FITC-labeled tubulin was able to polymerize into MTs to an extent identical to that of unlabeled tubulin in a buffer solution (4 M glycerol, 100 mM PIPES [pH 6.8], 5 mM $MgCl_2$, 1 mM EGTA, and 0.5 mM GTP) that supports tubulin polymerization without MAPs (data not shown). In contrast, when MTs preformed without MAPs were conjugated with FITC, the FITC-labeled tubulin showed a D/P ratio of from 0.25 to 0.3. Although the FITC-labeled tubulin showed lower polymerizability than did unlabeled tubulin (Fig. 1), MTs assembled from the labeled tubulin were clearly observed by fluorescence microscopy.

As measured by SDS-polyacrylamide gel electrophoresis, the FITC-labeled brain tubulin fraction contained no detectable amount of MAPs, even when it was heavily loaded (Fig. 2). Furthermore, a fluorescence band detected after electrophoresis corresponded to the position of tubulin with no fluorescence at the positions of MAPs.

Before the behavior of the endogenous tubulin in living cells is analyzed by injection of FITC-labeled porcine brain tubulin, it is desirable to examine whether the labeled tubulin can not only polymerize by itself but also be incorporated into endogenous MTs or co-polymerize with the endogenous tubulin. In the assay of such a co-polymerization, we used outer fiber tubulin purified from the starfish sperm instead of tubulin from the sand dollar egg itself because we had a big supply of the former. Curve *1* in Fig. 3 shows polymerization of outer fiber tubulin (1.1 mg/ml). When FITC-labeled porcine brain tubulin (final concentration, 0.6 mg/ml) was added to the outer fiber tubulin solution, resulting in 1.7 mg/ml of the total tubulin concentration, the rate of polymerization and the steady state level increased (curve *2*). When we replaced the FITC-labeled tubulin with unlabeled porcine brain tubulin, we observed similar co-polymerization; with the addition of unlabeled tubulin we attained a steady state level slightly higher than with the addition of labeled tubulin (curve *3*) but the level was still lower than that seen in polymerization of outer fiber tubulin alone at the same concentration of 1.7 mg/ml (curve *4*). These results indicate that the labeled porcine brain tubulin was competent to co-polymerize with outer fiber tubulin under this assembly medium condition. Because this condition was more favorable for outer fiber tubulin than for porcine brain tubulin (18, 21, 28), the decrease in the polymerization rate and the steady state level by mixing of porcine brain tubulin may be attributable to an increase in the critical concentration for polymerization of the brain tubulin in this assembly medium.

The co-polymerization of the labeled tubulin with outer fiber tubulin was also demonstrated by the centrifugation assay, as shown in Table I (compare Materials and Methods). As the ratio of FITC-labeled tubulin to outer fiber tubulin increased and the total tubulin concentration was held constant, total fluorescence of the sedimented MTs and the fluorescence intensity (B/A in Table I) increased, whereas the

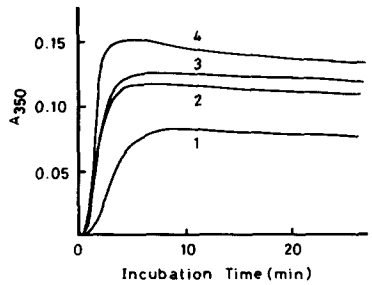

FIGURE 3 Co-polymerization of labeled porcine brain tubulin with outer fiber tubulin. Tubulin mixtures were polymerized as described in Materials and Methods. Curves *1* and *4* represent outer fiber tubulin alone, curve *2* outer fiber tubulin mixed with FITC-labeled tubulin at a molar ratio of 2:1, and curve 3 outer fiber tubulin mixed with phosphocellulose purified unlabeled tubulin at a molar ratio of 2:1. Tubulin concentrations were 1.1 mg/ml for curve *1* and 1.7 mg/ml for curves *2*, *3*, and *4*. Buffer condition was the same as that used in the low speed centrifugation assay.

amount of assembled MTs decreased consistently with the turbidimetry assay (data not shown). When FITC-labeled tubulin alone was incubated, only a trace amount of MTs showing high fluorescence intensity was sedimented under this assembly medium condition. In Table I, the series of the values for the total fluorescence and the fluorescence intensity of the supernatants at various mixing ratios show that a part of the labeled tubulin was co-polymerized with outer fiber tubulin, depending on their mixing ratio.

We also examined elongation of MTs when fragments of sperm flagellar axonemes were used as seeds, to which FITC-labeled brain tubulin was added. Fig. 4 demonstrates that several MTs grew from the axonemal doublet MTs as visualized by differential interference microscopy (*b*) and fluorescence microscopy (*a*). We should stress that the fluorescence image clearly shows that the labeled tubulin polymerized from both the axonemal MT ends and did not bind to the MT wall of the axonemal fragment even after a prolonged incubation (1 h).

Distribution of FITC-labeled Tubulin during Fertilization

When the FITC-labeled brain tubulin was injected into unfertilized sand dollar eggs, the fluorescence diffused uniformly over the entire cytoplasm within 5–10 min but did not enter a female pronucleus. The FITC-labeled tubulin had already polymerized to some extent before injection because fibrous structures were observed in injection solution in glass capillary reservoir at 25°C by differential interference microscopy. We think that the spreading rate of the FITC-labeled tubulin sample containing MTs may be slower than that of tubulin dimer itself. The egg cytoplasm contains many fluorescent particles with a color slightly different from that of FITC-labeled tubulin.

The injected eggs were fertilized after the labeled tubulin diffused uniformly. A series of differential interference and fluorescence micrographs of the same egg during fertilization directly recorded on 35-mm film are shown in Fig. 5. When a sperm aster began to form, fluorescence became intense at the region of the sperm aster (Fig. 5, *b* and *d*). As the aster grew, both the intense area and the intensity of the fluoresence of the sperm aster increased (Fig. 5*f*). Astral rays were detected to be fluorescent in the egg cytoplasm. The fluorescence intensity of the sperm aster decreased 10–15 min after fertilization, when the aster was still growing (Fig. 5, *i* and *j*). This decrease was not caused by photobleaching, because the fluorescence of the sperm aster was weak even when the sperm aster was observed by fluorescence microscopy for the first time 10–15 min after fertilization and because the fluorescence of the diaster and the mitotic apparatus became intense again. The fertilized eggs injected with FITC-labeled tubulin divided normally.

In the eggs injected with FITC-labeled BSA, the distribution of fluorescence remained uniform over the entire egg cytoplasm including the sperm aster. FITC-labeled BSA did not enter either the female pronucleus or the zygote nucleus.

TABLE I

Co-polymerization Assay by Low-speed Centrifugation

FITC-labeled porcine brain tubulin/outer fiber tubulin		Protein concentration (A)	Fluorescence (B)	B/A
		mg/ml		
1:5	Sup	0.34	3.8	11.2
	Ppt	1.13	4.4	3.9
2:4	Sup	0.43	5.5	12.8
	Ppt	1.05	6.1	5.8
3:3	Sup	0.61	10.3	16.9
	Ppt	0.77	6.8	8.8
6:0	Sup	1.48	31.8	21.5
	Ppt	0.08	2.2	27.5

FITC-labeled porcine brain tubulin was mixed with sperm outer fiber tubulin at various ratios and incubated for 20 min at 37°C as described in Materials and Methods. Fluorescence was measured by a fluorospectrophotometer (Hitachi 650-10 S; Hitachi Ltd., Tokyo) with excitation and emission wave lengths of 495 and 520 nm, respectively. Fluorescence in the table is represented by relative value. Sup and Ppt, supernatant and precipitate, respectively.

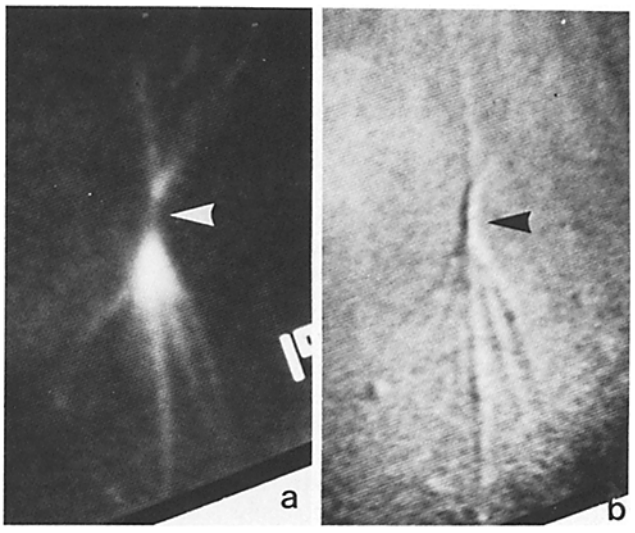

FIGURE 4 A set of fluorescence (*a*) and differential interference (*b*) micrographs of a fragment of sea urchin sperm axoneme. Micrographs were taken with the video camera ~60 min after incubation of the fragment in PMEG and 4 mg/ml of FITC-labeled brain tubulin at 32°C. Several fluorescent MTs were polymerized from both ends of the axonemal fragment as a seed. The arrowheads indicate the fragment. × 3,000.

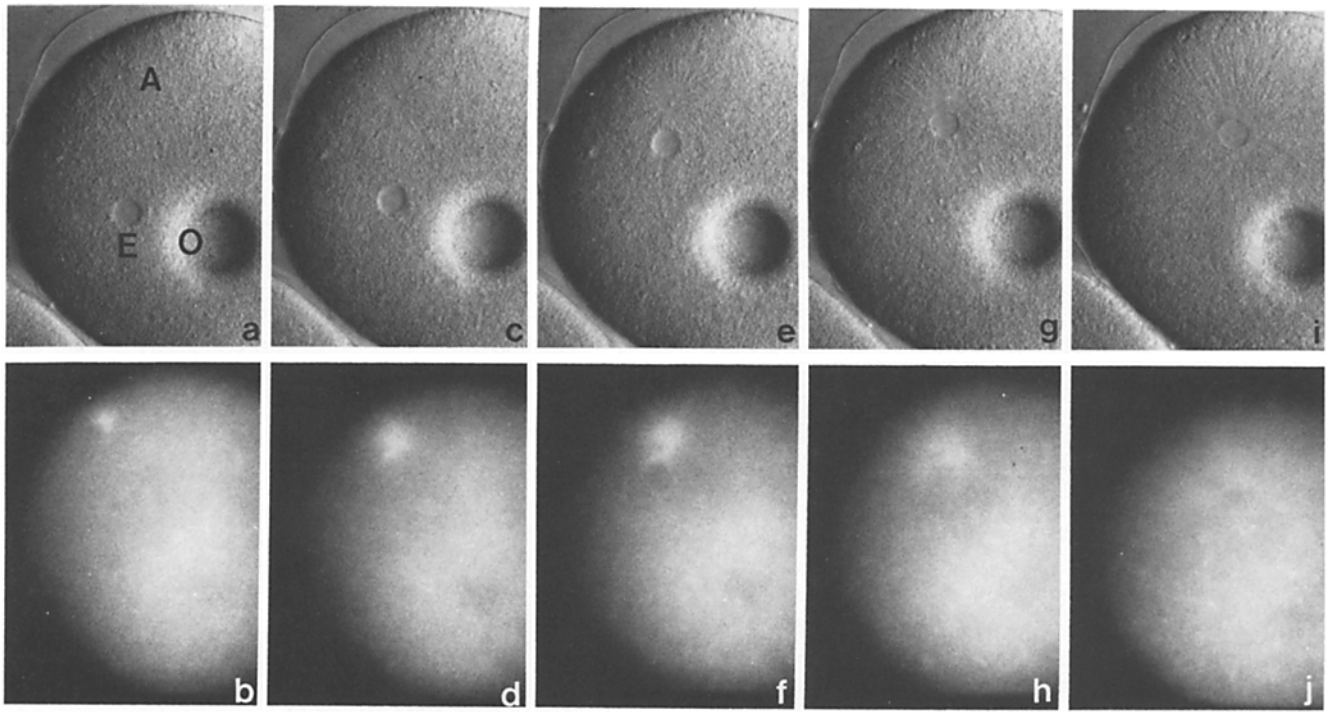

FIGURE 5 Distribution of FITC-labeled brain tubulin in an egg during fertilization. Fluorescence micrographs are shown in the lower row (*b*, *d*, *f*, *h*, and *j*) and differential interference micrographs in the upper row (*a*, *c*, *e*, *g*, and *i*). They were recorded directly on 35-mm film. Injection was carried out 10 min before insemination. (*a* and *b*) 5 min 30 s; (*c* and *d*) 8 min; (*e* and *f*) 9 min; (*g* and *h*) 9 min 40 s; (*i* and *j*) 15 min 40 s after insemination. *a* and *b* were taken just after the sperm aster was detected. In *e* and *f*, migration of egg pronucleus is shown. *A*, sperm aster; *E*, egg pronucleus; *O*, oil drop introduced into the egg at the time of microinjection. × 500.

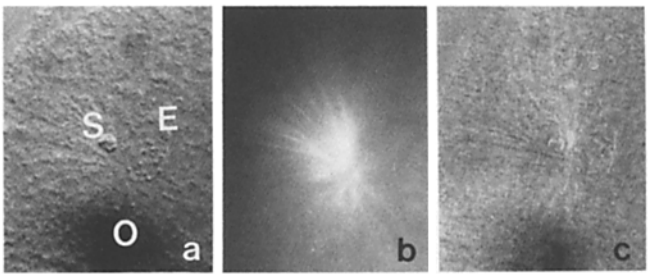

FIGURE 6 A set of differential interference (*a*), fluorescence (*b*), and polarization (*c*) micrographs of an egg injected with FITC-labeled brain tubulin, fertilized, and lysed shortly after the egg pronucleus arrived at the center of the sperm aster. Microinjection was carried out >10 min before insemination. *E*, egg pronucleus; *O*, oil drop introduced into the egg at the time of microinjection; *S*, sperm pronucleus. × 650.

When some eggs injected with FITC-labeled tubulin were lysed ~10 min after fertilization, the structures of the sperm aster were preserved and stabilized as shown in Fig. 6. The same fibrous structures of the sperm aster are observed by differential interference, fluorescence, and polarization microscopy. The fluorescence images demonstrate that the FITC-labeled tubulin is distributed along the astral fibers, and are consistent with the labeled tubulin's being incorporated into the astral MTs.

Distribution of FITC-labeled Tubulin during Mitosis

PORCINE BRAIN TUBULIN: When FITC-labeled brain tubulin was injected into fertilized eggs at streak stage, the labeled tubulin was incorporated rapidly into structures around the nucleus, especially into the central regions of growing asters. When the injected region was near one of the asters, the fluorescence was more intense at that aster than at the other during a short period after the injection. The FITC-labeled tubulin diffused uniformly within 5–10 min after injection except for in the nucleus and the surrounding cytoplasmic layer, with two asters around the nucleus.

When the injected volume was >5% of the egg volume, streak stage persisted for a long period. The astral rays grew long and thick, and the nucleus was stretched abnormally and sometimes divided into two. Accordingly, the normal mitotic apparatus did not appear and then cleavage did not occur. When the volume was 3–5% of the egg volume, the small mitotic apparatus was formed and its birefringence was weak. Therefore, the cleavage was not always completed. When the volume was <3% of the egg volume, the mitotic apparatus was formed normally. The injected eggs underwent cleavage, but in some cases the mitotic spindle was shorter than the normal one. The inhibitory effect of the injection on the mitotic spindle appeared to depend on the amount of the injected tubulin rather than on the injection volume.

Similar changes in the distribution of FITC-labeled tubulin were repeated during the first several cleavage cycles. This fact indicates that the FITC-labeled brain tubulin injected into sand dollar eggs was used repeatedly and was fairly stable for a long period.

Fig. 7 shows a series of differential interference, fluorescence, and polarization micrographs recorded with a video camera during second mitotic cycle. Stable fluorescent figures with little photobleaching are displayed throughout cleavage on the fluorescence micrographs in which the time the blas-

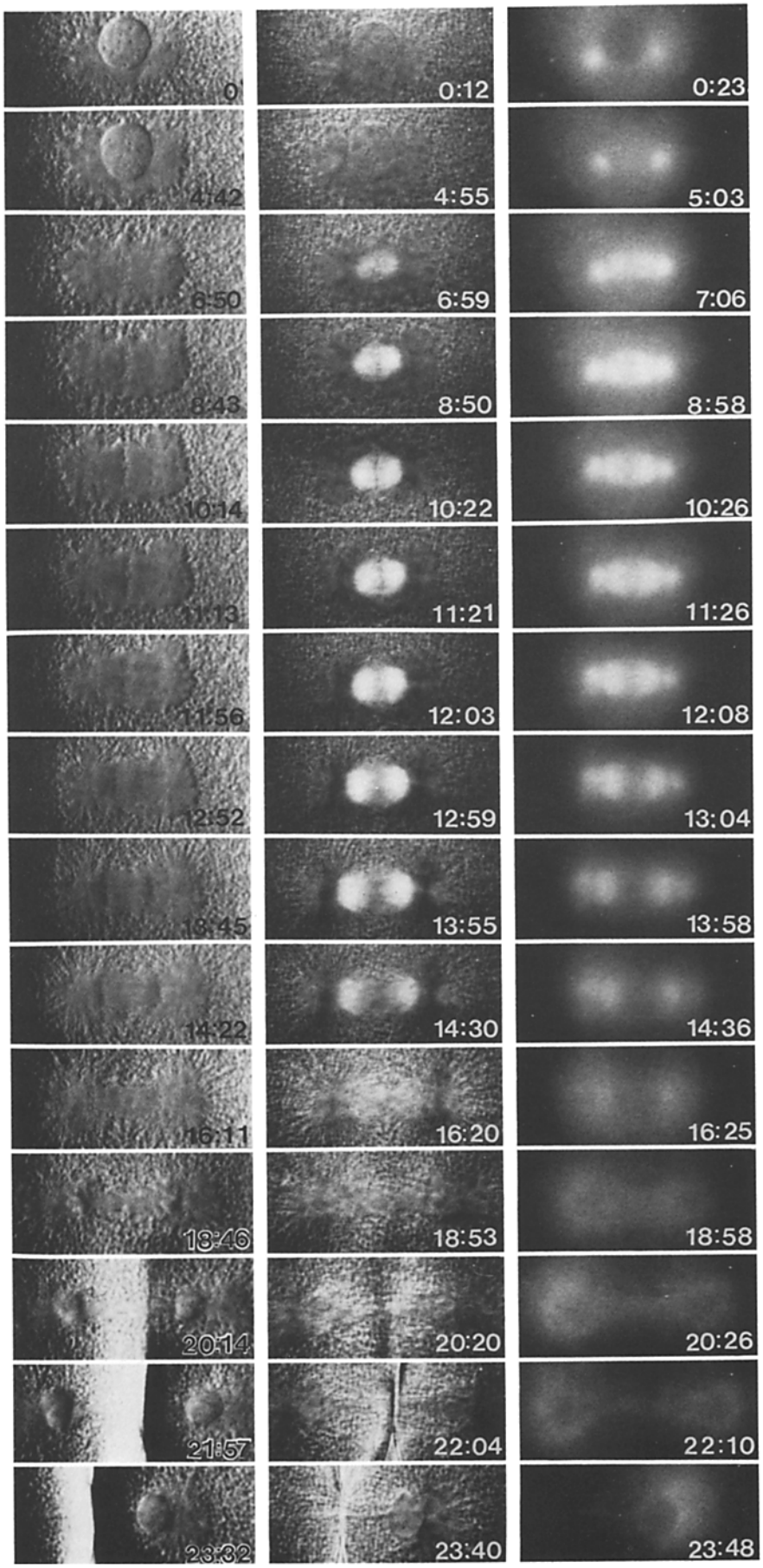

FIGURE 7 Distribution of FITC-labeled brain tubulin in a blastomere through the second cleavage. Injection was carried out before the first cleavage. Fluorescence micrographs are in the right column, polarization micrographs are in the middle column, and differential interference micrographs are in the left column. Differential interferences, polarization, and fluorescence micrographs were recorded in this sequence with the video camera. Numbers on the micrographs are the times (minutes: seconds) after the first micrograph was taken. × 520.

tomere was exposed to the excitation light was short. Differential interference micrographs showing the nuclei and chromosomes in the left column in Fig. 7 indicate the mitotic stages of the blastomere. When the nuclear envelope disappeared, the fluorescence gradually became apparent at the region between the intensely fluorescent asters forming a shape of the spindle. The fluorescence of the mitotic spindle became intense with time. The distribution of the fluorescence of the spindle at various stages of mitosis was usually the same as those of birefringence, although a trough of the fluorescence intensity at the spindle equator appeared steeper than that of the birefringence at the same region. In contrast, asters observed by fluorescence microscopy were more noticeable than those observed by polarization microscopy. The difference can be ascribed to the fact that the birefringence is derived from well-oriented MTs whereas the fluorescence is independent of their orientation.

The fluorescent region in the mitotic apparatus appeared to consist of four parts, two asters and two half-spindles. During metaphase, the fluorescence of the astral centers was more intense than that of the spindle. From metaphase to early anaphase the fluorescence intensity of the asters was more or less constant, whereas the area of intensely fluorescent region increased. During anaphase, the fluorescence of the spindle reached a maximum and then decreased with increasing spindle length. During midanaphase and telophase the intense fluorescence in the astral centers was dividing into two, and the fluorescent region in the aster became larger and larger, and the fluorescence was weak at the equatorial region. When the daughter nuclei appeared, two fluorescent spots were detected clearly near each nucleus, and they became astral centers in the next mitosis. During interphase, the fluorescence was more or less uniform over the entire blastomeres, except for the spots near the nucleus mentioned above.

Fig. 8, *a* and *b* show fluorescence and differential interference micrographs of a normal uninjected egg. There are many autofluorescent particles in the egg cytoplasm. Because autofluorescent particles are excluded from the yolk-free region where the mitotic apparatus forms, the same region in uninjected eggs is less fluorescent than the surrounding egg cytoplasm during mitosis. The fluorescence of the uninjected egg was far less intense than those of the eggs injected with fluorescently labeled proteins, although the egg in Fig. 8*b* whose exposure was controlled automatically seemed to be as intensely fluorescent as the injected eggs.

As shown in Fig. 8*d*, injected FITC-labeled BSA was slightly concentrated in the yolk-free region during mitosis, but structures characteristic of the spindle and asters were not recognized in that region by fluorescence microscopy. Injected FITC-labeled denatured tubulin appeared to remain in the granular egg cytoplasm outside the mitotic apparatus (Fig. 8*f*). FITC-labeled denatured tubulin diffused more slowly than FITC-labeled native tubulin and was concentrated in the punctate pattern surrounding the mitotic apparatus. This result indicates that the tubulin loses the ability to be incorporated into microtubular structures in the eggs when it is denatured.

The eggs injected with FITC-labeled tubulin were lysed at desired stages after injection. Lysis was carried out >10 min after injection when the fluorescence diffused evenly over the entire egg cytoplasm. Figs. 9–13 show the mitotic structures from prophase through telophase of the injected and lysed eggs. In all of the figures the fibrous structures are demonstrated more clearly than those in living eggs. These structures

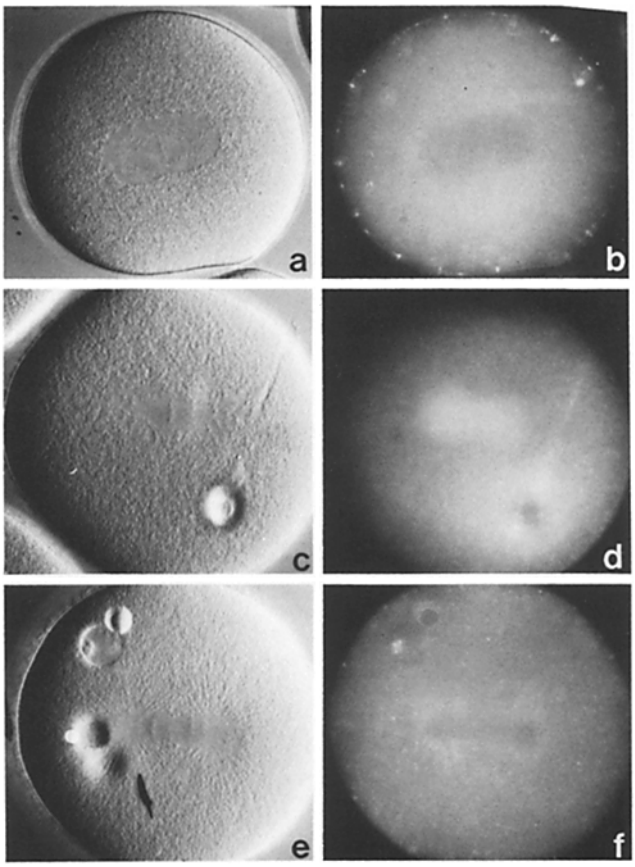

FIGURE 8 Corresponding differential interference and fluorescence micrographs of mitotic eggs in control experiments. (*a* and *b*) A normal egg at metaphase. The fluorescence of the uninjected egg (*b*) was much less intense than that of the other control eggs (*d* and *f*), though the fluorescent intensity of these eggs appears equivalent to that of the uninjected egg because the exposure time for the uninjected egg was very long. (*c* and *d*) An egg at metaphase 2 min 40 s after the injection of the FITC-labeled BSA, still diffusing unevenly. (*e* and *f*) An egg at late anaphase 41 min after the injection of FITC-labeled denatured tubulin. The denatured tubulin was concentrated in the punctate pattern surrounding the mitotic apparatus. × 300.

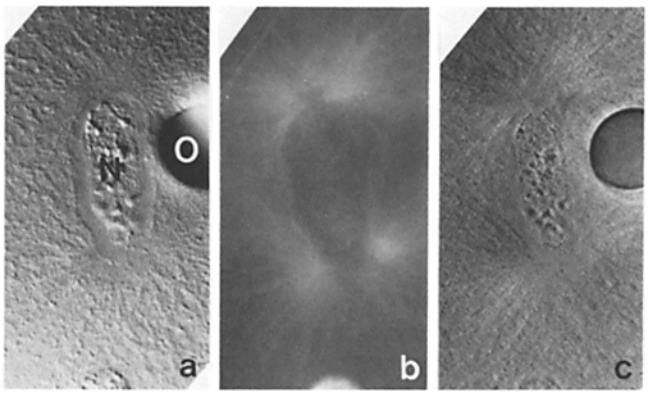

FIGURE 9 A set of differential interference (*a*), fluorescence (*b*), and polarization (*c*) micrographs of an egg lysed at prophase after injection of FITC-labeled tubulin. *N*, Nucleus; *O*, oil drop introduced into the egg at the time of microinjection. × 570.

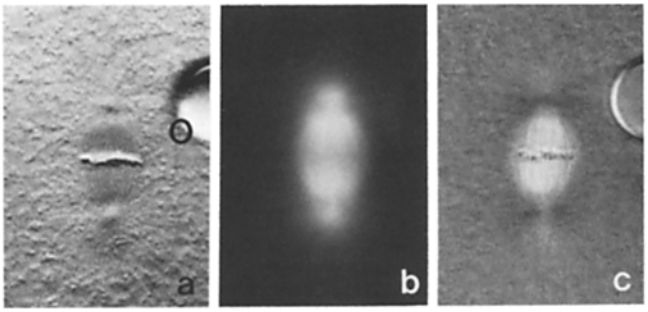

FIGURE 10 A set of differential interference (*a*), fluorescence (*b*), and polarization (*c*) micrographs of an egg lysed at metaphase after injection of FITC-labeled tubulin. *O*, oil drop introduced into the egg at the time of microinjection. × 700.

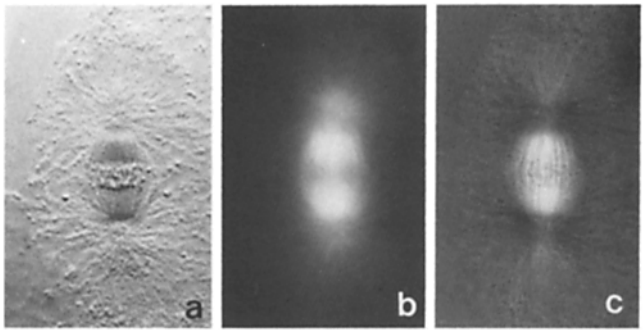

FIGURE 11 A set of differential interference (*a*), fluorescence (*b*), and polarization (*c*) micrographs of an egg lysed at early anaphase after injection of FITC-labeled tubulin. × 700.

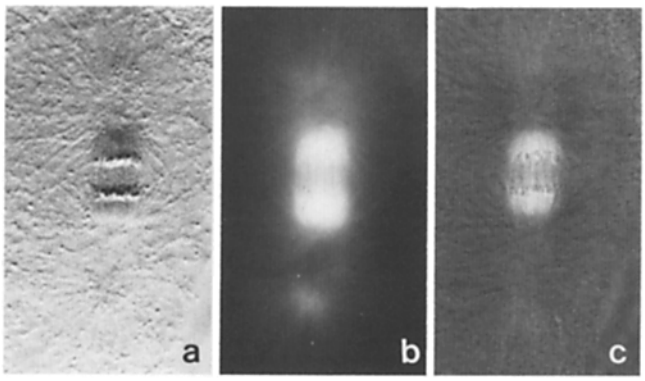

FIGURE 12 A set of differential interference (*a*), fluorescence (*b*), and polarization (*c*) micrographs of an egg lysed at midanaphase after injection of FITC-labeled tubulin. × 700.

visualized by fluorescence microscopy coincided with those by differential interference and polarization microscopy. The results from this experiment strongly confirm the conclusion obtained in living cells that the FITC-labeled brain tubulin is incorporated into MTs in the mitotic apparatus. The fluorescence in the astral centers of the lysed specimen appeared less intense than in those in living cells. This result suggests that the centers contain some diffusible tubulin polymers, oligomers, and/or dimers that would leave the centers during the lysis procedure.

MT PROTEINS: When the FITC-labeled tubulin was replaced with FITC-labeled MT proteins for injection, identical fluorescence patterns were observed during mitosis, although the fluorescence of the mitotic apparatus was not so distinct

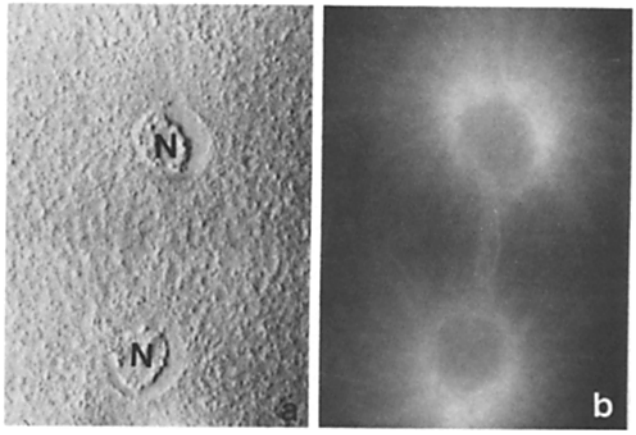

FIGURE 13 A set of differential interference (*a*) and fluorescence (*b*) micrographs of an egg lysed at telophase after injection of FITC-labeled tubulin. *N*, Daughter nuclei. × 700.

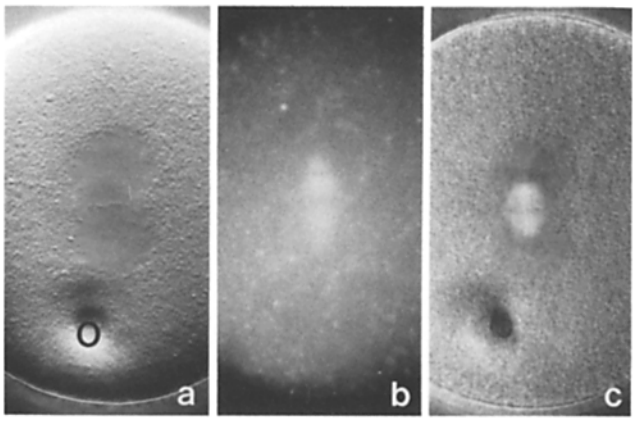

FIGURE 14 A set of differential interference (*a*), fluorescence (*b*), and polarization (*c*) micrographs of an egg at metaphase after injection of FITC-labeled outer fiber tubulin. The micrographs were taken 31 min after the injection. One of the asters is out of focus because the spindle axis is not exactly horizontal. Fluorescent particles in the cytoplasm surrounding the mitotic apparatus are not aggregates of FITC-labeled tubulin but autofluorescent particles. *O*, Oil drop introduced into the egg at the time of microinjection. × 400.

from the cytoplasmic background (photographs not shown). In the case of the FITC-labeled MT proteins, MAPs were labeled more heavily than tubulin and had a relatively low protein concentration (4 mg/ml). That MAPs were shown not to be incorporated into microtubular fibrous structures in living cells (16) also explains the weak fluorescence of the mitotic apparatus described above.

STARFISH SPERM TUBULIN: When purified axonemal tubulin labeled with FITC was injected into fertilized eggs, localization pattern of the tubulin (Fig. 14) was identical to that observed when FITC-labeled brain tubulin was used. However, the fluorescence of the mitotic apparatus was weaker than that in the latter case, which may be attributable to the lower concentration of the axonemal tubulin (4 mg/ml).

Incorporation Rate of FITC-labeled Tubulin into the Mitotic Apparatus

The demonstration that the assembly-competent heterogeneous tubulin is used to construct the mitotic apparatus

without apparent discrimination from egg tubulin led us to design the experiments to determine whether or not the labeled heterogeneous tubulin is incorporated into or exchanged with tubulin in MTs of the *preformed* mitotic apparatus. For this purpose the labeled tubulin was injected into metaphase or anaphase eggs.

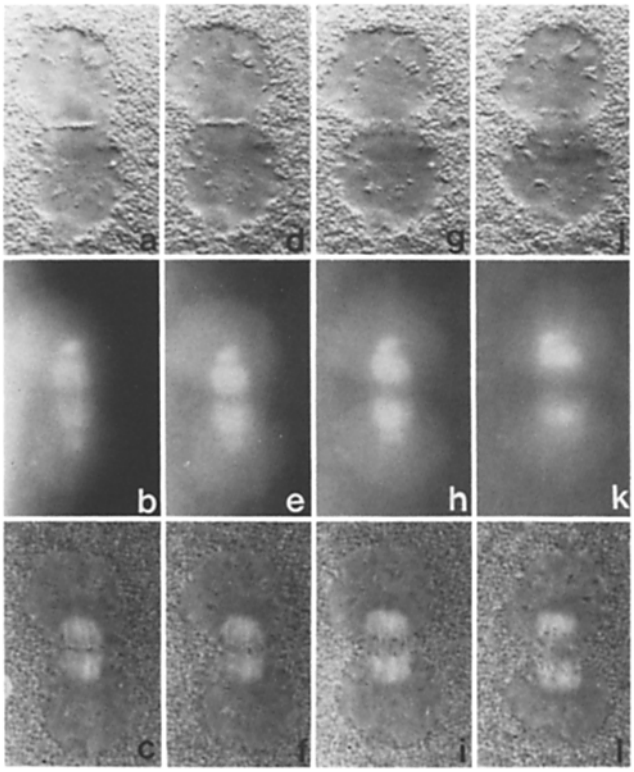

FIGURE 15 Time course of the incorporation of FITC-labeled brain tubulin into a metaphase mitotic apparatus after injection at a position on the extension of the equatorial plane. Corresponding differential interference (*a*, *d*, *g*, and *j*), fluorescence (*b*, *e*, *h*, and *k*), and polarization (*c*, *f*, *i*, and *l*) micrographs are in the upper, middle, and lower rows, respectively. They were recorded directly on 35-mm film. The fluorescence micrographs (*b*, *e*, *h*, and *k*) were taken at 55 s, 2 min, 3 min, and 5 min 20 s after injection, respectively. × 500.

When we injected the labeled tubulin at a region 30 μm apart from the spindle axis on the extension of the equatorial plane (Fig. 15), the fluorescently labeled tubulin was incorporated fairly rapidly into both the spindle and the asters as soon as the concentration of the labeled tubulin increased by diffusion in the surrounding cytoplasm. Note in this figure that the incorporated area in the spindle spreads from left to right with a straight boundary (compare Fig. 15*b*), indicating that the incorporation occurs almost simultaneously within the same spindle fiber. Fluorescence intensity increased in parallel over the same half-spindle as the concentration of the labeled tubulin increased by diffusion in the surrounding cytoplasm. The mode of incorporation of FITC-labeled tubulin in the anaphase mitotic apparatus was almost the same as that in the metaphase one mentioned above.

When the labeled tubulin was injected 20 μm away from a pole on the extension of the spindle axis (Fig. 16), the fluorescence rapidly spread in a direction longitudinal of the mitotic apparatus. Note in the photograph at 29 s after injection that fluorescence intensity is more or less uniform within each half-spindle whereas it is distinctly different between two half-spindles. Note also that the fluorescence intensity of the lower half-spindle definitely increases within a period from 29 to 41 s and is more or less uniform in both the 29- and the 41 s half-spindle. The above result strongly indicates that the incorporation of tubulin occurs almost simultaneously over the same half-spindle.

No change in birefringence was detected by injection of FITC-labeled tubulin. In some cases, the spindle shortened a few minutes after injection and did not elongate to normal extent during anaphase.

DISCUSSION

Distribution of FITC-labeled Tubulin in MT Structures

The present study shows that FITC-labeled heterogeneous tubulin injected into sand dollar eggs is localized in MT-containing structures such as the sperm aster during fertilization and the mitotic apparatus during the successive cleavages. This result is in contrast to the result by Wadsworth and Sloboda (42) that 5-(4,6-dichlorotriazin-2-yl)aminofluores-

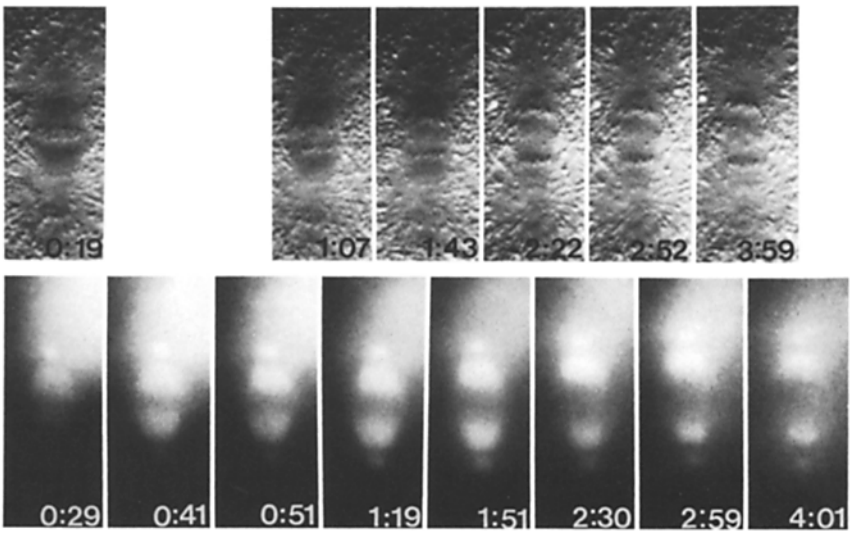

FIGURE 16 Time course of the incorporation of FITC-labeled brain tubulin into an anaphase mitotic apparatus after injection at a position on the extension of the spindle axis. Differential interference and fluorescence micrographs recorded with the video camera are in the lower and upper rows, respectively. The numbers on the micrographs indicate the times (minutes:seconds) after injection. × 520.

cein-labeled tubulin was not incorporated into the mitotic spindle. However, the results reported by Salmon et al. (35) and Saxton et al. (36), using DTAF-labeled tubulin, appear to be consistent with the present study. The fibrous structures of the sperm aster, the mitotic apparatus, and the region around the nucleus in lysed cells visualized by fluorescence microscopy coincided exactly with those observed by polarization and differential interference microscopy. This fluorescence microscopic observation with the lysed cells is consistent with those obtained by immunofluorescence microscopy (1, 7).

It is evident that the sand dollar egg cannot completely distinguish the injected porcine brain and starfish sperm tubulin from its own tubulin. In other words, these heterogenous tubulins can function in mitosis as endogenous egg tubulin. Moreover, the co-assembly into the mitotic structures did not at all affect mitosis or even cytokinesis at low concentrations of the injected tubulin, though the FITC-labeled heterogeneous tubulins did not appear to assemble as efficiently as the endogenous tubulin in the egg. This means that even brain tubulin, which would play a role in the axonal transport or nerve excitation (24), preserves its function in mitosis.

In the present study, a distinct gap was found between the spindle and central parts of asters (centrosphere), which suggests that the MT organization is discontinuous in the regions connecting the spindle with the asters. This is consistent with the observation that the asters of the glycerol-isolated mitotic apparatus are easily separable from the spindle by gentle shaking (34). This region was previously shown to be a locus for accumulation of fluorescently labeled calmodulin, although the physiological meaning is not yet known (5, 6).

Fluorescence and Birefringence

In the present study, both of the intensity of birefringence and the size of the mitotic apparatus were unchanged by the injection of the FITC-labeled tubulin at low concentrations (1–3% of the egg volume), though the labeled tubulin was accumulated in the spindle. The tubulin concentration by the injection of FITC-labeled tubulin solution at 3% of the egg volume increased by 0.2–0.3 mg/ml in the egg cytoplasm in the present study. If all of the injected tubulins are competent to assemble into MTs and if the concentration of tubulin originally present in the egg is 0.57 mg/ml, as calculated from a value of 1.2×10^{-4} μg soluble MT protein/egg (30) for an egg of diameter 74 μm (8), then the tubulin pool would increase by ~50% at a maximal estimation by the injection of FITC-labeled tubulin; this makes equilibrium shift, thereby causing augmentation of birefringence. However, it was not the case in the present study. If the egg tubulin pool is, for example, 5 mg/ml (3), the increase in tubulin pool is calculated to be only 5% after injection. This may cause little increase in birefringence. In addition, the co-polymerization experiments suggest that the co-polymerizability of FITC-labeled porcine brain tubulin with egg tubulin may be lower than the polymerizability of egg tubulin itself in the egg cytoplasm at a low temperature (25°C) and a high ionic strength. Moreover, the high concentrations of the injected tubulin resulted in the decrease in both the intensity of birefringence and the size of the mitotic apparatus. The absence of the increase in birefringence of the spindle by microinjection of tubulin may be due to the difference in the condition between in vitro and in vivo.

Incorporation Rate of FITC-labeled Tubulin

FITC-labeled tubulin was rapidly incorporated into the mitotic apparatus soon after injection into metaphase or anaphase eggs. The fluorescence intensity was uniform within each half-spindle as compared with that in the surrounding cytoplasm (Figs. 15 and 16). The possibility that the rapid incorporation was due to quick adsorption of denatured tubulin in FITC-labeled tubulin preparation to the spindle appeared to be excluded by the result that denatured tubulin failed to be accumulated in the mitotic apparatus. The rapid incorporation might be due to the possibility that new MTs are being formed in the spindle region or that the FITC-labeled tubulin is binding to the walls of the spindle and astral MTs by an unknown tubulin-specific binding mechanism. However, the birefringence of the spindle did not increase after the addition of FITC-labeled tubulin, and the labeled tubulin was polymerized from the ends of the axonemal seeds instead of binding along the axoneme. These observations argue against both of the above possibilities but do not rule them out.

If the injected tubulins were incorporated from the fast-growing end (plus end) of each MT located at a particular region (e.g., equatorial region) of the spindle and transmitted along it at a rate of ~1 μm/min as expected from in vitro study (2), a spread of fluorescence initiated at the equatorial region of the spindle would be observed after microinjection of FITC-labeled tubulin. This is not the case. The result of the present study indicates that the incorporation of tubulin occurs almost simultaneously over the half-spindle. We interpret this fact to mean either that the transmission rate of incorporated tubulin in the MT is higher than the rate determined by in vitro experiments or that the tubulin is not incorporated at a particular end but over the entire length of MTs. The latter possibility of exchange of tubulin dimers between assembled MTs and free dimers has been suggested by Suzaki et al. (40) from an in vitro experiment and by Salmon et al. (35) from an in vivo experiment.

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