Buffer Conditions and Non-Tubulin Factors Critically Affect the Microtubule Dynamic Instability of Sea Urchin Egg Tubulin

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The dynamic instability of individual microtubules (Mts) in cytoplasmic extracts or assembled from highly purified sea urchin egg tubulin was examined using video-enhanced, differential-interference contrast (VE-DIC) light microscopy. Extract Mts (endogenous tubulin = 12.1 μM) displayed only plus-ended growth. The elongation velocity was 7.8 μm/min for an average duration of 1.3 min before switching (catastrophe) to rapid shortening, which occurred at 13.0 μm/min for an average duration of 0.5 min before switching (rescue) back to the elongation phase. These parameters are typical of interphase Mt dynamic instability. Surprisingly, Mts assembled from purified urchin egg tubulin in standard buffers were less dynamic that those reported for purified brain tubulin or Mts in the extract. Buffer parameters were changed in an attempt to mimic the extract Mt results. The pH buffer itself, Hepes or Pipes, drastically altered Mt dynamics but could not achieve high elongation velocity with high catastrophe frequencies. Calcium at 1 μM had negligible effects, while increasing pH from 6.9 to 7.2 stimulated elongation velocity. Finally, Mt dynamics of purified egg tubulin (11.9 μM) were assayed in ultrafiltrates (MW cut-off <30 kD) of the cytoplasmic extracts. Mts elongated slowly at 1.2 μm/min for 26 min before a catastrophe and rapid shortening at 11.8 μm/min. Rescue was less frequent than unfiltered extracts, minus-ended growth was observed, and self-assembly occurred at slightly higher tubulin concentrations. Therefore, the egg extracts and cytoplasm must contain non-buffer factors which stimulate elongation velocity by 6.5-fold without self-assembly, increase catastrophe frequency by 20-fold, and block minus-ended growth.

Key words: Pipes, Hepes, calcium, VE-DIC microscopy, cytoplasmic extracts

INTRODUCTION

The fundamental mechanism of microtubule assembly in the cytoplasm is dynamic instability [Cassimeris et al., 1988; Schulze and Kirschner, 1987, 1988; Sammak and Borisy, 1988; Chen and Schliwa, 1990; Hayden et al., 1990]. Following nucleation from the centrosome in animal cells, the great majority of microtubules (Mts) elongate by tubulin subunit addition to their plus ends oriented distal from the centrosome [Telzer and Haimo, 1981]. The ends can persist for variable periods of time in elongation until they have an abrupt transition (catastrophe) into a rapid shortening phase. An end can persist in rapid shortening until either it switches back to elongation (rescue) or it completely disassembles. In interphase and mitosis, the reported velocities of elongation range from 4 μm/min in mammalian culture cells [Schulze and Kirschner, 1988] to 7–18 μm/min in newt lung epithelial cells [Cassimeris et al., 1988; Hayden et al., 1990] and extracts of Xenopus and sea urchin egg.

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Abbreviations used: MAPs, microtubule-associated proteins; Mts, microtubules; VE-DIC microscopy, video enhanced, differential-interference contrast light microscopy

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cytoplasm [Gard and Kirschner, 1987; Belmont et al., 1990; Verde et al., 1990] (Glikson and Salmon, in preparation). The corresponding velocities of rapid shortening are on the order of 15–20 μm/min. Cellular changes between interphase and mitosis produce a large reduction (up to 10-fold) in average length between the Mts of the interphase cytoplasmic microtubule complex (CMT) and the mitotic spindle [Cassimeris et al., 1987]. These changes may involve an increase in catastrophe frequency [Belmont et al., 1990] and a decrease in rescue frequency [Cassimeris et al., 1988]. (Glikson and Salmon, in preparation). Nevertheless, Mts in both interphase and mitosis have high frequencies of catastrophe (1/min or less) at fast growth velocities.

In contrast, detailed analysis of the dynamic instability properties of Mts assembled from purified brain tubulin [Walker et al., 1988; O’Brien et al., 1990; Mitchison and Kirschner, 1984; Horio and Hotani, 1986] has shown that under the conditions tested, the Mts do not have the simultaneous fast growth velocities and high catastrophe frequencies that are observed in living cells, whether in interphase or mitosis, or in cytoplasmic extracts capable of supporting Mt polymerization [Schulze and Kirschner, 1988; Sammak and Borisy, 1988; Cassimeris et al., 1988; Hayden et al., 1990; Gard and Kirschner, 1987; Verde et al., 1990; Belmont et al., 1990] (Glikson and Salmon, in preparation). One possibility for this result could be that there are intrinsic differences in the dynamic instability of tubulin isolated from different sources, especially considering that brain tubulin is from a terminally differentiated cell. This possibility is supported by the fact that Mts are relatively static in neural cells [Okabe and Hirokawa, 1988; Seitz-Tutter et al., 1988], and there are differences between the bulk assembly properties of purified brain vs. non-neuronal tubulin such as sea urchin egg [Detrich et al., 1985; Suprenant and Rehbn, 1983]. Spisula solidissima egg [Suprenant and Rehbn, 1984], and chick erythrocytes [Murphy and Wallis, 1983]. However, there has been no information on the dynamic instability of Mts assembled from any non-neuronal source. The differences may also be due to non-tubulin factors such as ambient ionic conditions or microtubule-associated proteins (MAPs).

The purpose of this study was to establish the degree that Mt dynamic instability depends upon the intrinsic properties of the tubulin species vs. non-tubulin factors. This was accomplished by first establishing the dynamic instability parameters of Mts assembled at 25°C in cytoplasmic extracts of unfertilized Strongylocentrotus purpuratus sea urchin eggs by video-enhanced, differential-interference contrast (VE-DIC) light microscopy. Sea urchin eggs contain a large pool of tubulin which is used for cytoplasmic Mt assembly in the developing embryo. Mt assembly in S. purpuratus egg extracts exhibits the rapid growth, rapid shortening, frequent catastrophe, and frequent rescue typical of interphase cytoplasm. Surprisingly, the Mts assembled from highly purified sea urchin egg tubulin in standard brain Mt reassembly buffers [e.g., Walker et al., 1988; Johnson and Borisy, 1977; Flynn and Purich, 1987] were significantly more stable than in cytoplasmic extracts and more stable than brain tubulin assembly in a similar reassembly buffer. Buffer parameters were altered to mimic physiological pH, calcium, and magnesium levels in an attempt to achieve cytoplasmic Mt dynamics. Finally, the exact buffer conditions of the active cytoplasmic extracts were examined by cycling purified urchin egg tubulin into cytoplasmic ultrafilters (MW cut-off <= 30 kD). We conclude from our results that the intrinsic properties of the tubulin and ambient ionic conditions of the cytoplasm cannot account for the fast elongation velocities or the high catastrophe frequencies found in living cells.

MATERIALS AND METHODS
S. purpuratus Cytoplasmic Extracts and Filtrates

Cytoplasmic extracts from S. purpuratus eggs were prepared according to Glikson and Salmon (in preparation) as follows. Unfertilized eggs were washed once in isotonic 19:1, then into isotonic HEMG lysis buffer (180 mM Hepes, 10 mM MgCl₂, 10 mM EGTA, 0.5 M glycine, pH 7.35) and packed for 3 min at 160 × g. Excess buffer was aspirated and 15% vol/vol lysis buffer was added back to the dejellied eggs (~5 ml). A final concentration of 2 mM GTP, 1 mM ATP, and 1 mM DTT, plus saturated PMSF, 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin protease inhibitors were added. The eggs were then homogenized just sufficiently to lyse the majority of eggs in a 7 ml glass/glass Dounce homogenizer on ice. The extract was sedimented at 50k × g for 45 min at 4°C in a TLA100.3 rotor (Beckman, Palo Alto, CA), and the middle clear layer was removed with a 22 gauge needle/syringe. The collected supernatant was centrifuged in a TLS55 rotor (Beckman) at 140k × g for 1 h at 4°C, and the clear layer was collected. This supernatant was snap-frozen in liquid nitrogen and stored at −80°C.

Endogenous tubulin concentration in the cytoplasmic extracts was determined by Western immunoblotting according to Towbin et al. [1979] using purified S. purpuratus tubulin as a standard. Blots were probed with monoclonal antibodies against S. purpuratus α-tubulin [Scholey et al., 1984] and HRP-conjugated goat antimouse secondary antibody (Hyclose Laboratories, Inc., Logan, UT), then developed with 4-chloro-1-naphthol (Sigma Chemical Co., St. Louis, MO). Quantitation was performed using a computing/laser densitometer model 300A (Molecular Dynamics, Sunnyvale, CA).
Mt Dynamic Instability of Sea Urchin Egg Tubulin

Cytoplasmic filtrates were prepared from the egg extracts as follows. A sample of extract was thawed and filtered in a centricron-30 device (Amicon Corp., Danvers, MA) by centrifugation at 3k × g in an SS-34 rotor (Sorvall, Wilmington, DE) for ~1 h. The retentate was discarded, and ultrafiltrate was collected and snap-frozen in liquid nitrogen and stored at ~80°C.

Preparation of Sea Urchin Egg Tubulin

Sea urchin egg tubulin was prepared from unfertilized sea urchin eggs (S. purpuratus) obtained from Marinus, Inc. (Long Beach, CA), essentially according to Suprenant and Marsh [1987] and Keller and Rebhun [1982] as detailed by Dr. R.E. Palazzo (personal communication) as follows. The initial 2-cycled tubulin steps were performed according to Suprenant and Marsh [1987] with slight modifications. Sea urchin eggs (~25 female urchins) were shed, by intracoelomic injection of 0.57 M KCl, into artificial sea water (Instant Ocean, Aquarium Systems, Eastlake, OH). The eggs were filtered through four layers of cheesecloth, then through a 118 μm polyester screen (Tetko, Inc., Briarcliff Manor, NY). The eggs were washed in seawater 2 times, dejelled in isotonic 19:1 (0.5 M NaCl, 27 mM KCl, 2 mM EDTA, pH 7.8), then resuspended to a 1:1 vol egg/lysis buffer (~100 ml dejelled eggs; 0.1 M Pipes, 1 mM MgSO4, 10 mM EGTA, 2 mM DTT, pH 7.3 at r.t.). The eggs were lysed using six to seven passes in a motor-driven glass/teflon homogenizer, or just sufficient to lyse most of the eggs. The extract was sedimented at 150k × g for 1 hr at 4°C, and the supernatant was collected and filtered through glass wool to remove lipids. The cytosolic extract was brought to 0.5 mM GTP and 8% DMSO and warmed to 25°C for 30 min to induce tubulin polymerization. Mts were sedimented at 37k × g, 40 min, 25°C, and the supernatant was aspirated and discarded. The Mts were resuspended in <1/5 the original cytosolic volume in PME buffer (0.1 M Pipes, 2 mM EGTA, 1 mM MgSO4, 1 mM GTP, pH 6.9 at r.t.) and depolymerized on ice with stirring for 30 min, and Dounce homogenized. The sample was clarified at 150k × g, 4°C, 30 min, and the supernatant was repolymerized by incubation at 30°C for 30 min. The Mts were sedimented at 25°C, the supernatant was aspirated and discarded, and the 2-cycled Mt pellets were stored at ~80°C until further use (up to several months). The DEAE purification steps were performed according to Keller and Rebhun [1982] as follows. The tubulin pellet was quickly defrosted, then depolymerized on ice for 30 min in DEAE buffer (10 mM Pipes, 1 mM MgSO4, 0.1 mM GTP, 0.02% sodium azide, pH 6.8 at r.t.). The sample was clarified at 150k × g, 4°C, 30 min, then applied to a 1.5 × 7 cm DEAE column (DE52; Whatman Biosystems Ltd., England). The column was washed with 0.3 M KCl in DEAE buffer, then the tubulin was stepped off with 0.6 M KCl. Fractions of A280nm >0.5 were pooled, then desalted on PD10 desalting columns (Sephadex G-25) according to the manufacturer’s instructions (Pharmacia, Uppsala, Sweden) using PME buffer as the eluant. Solid monosodium L-glutamate was added to the pooled/desalted tubulin to 1 M and dissolved on an ice bath, then polymerization was induced by warming to 37°C for 20 min. The Mts were sedimented at 60k × g, 25°C, 30 min, and the supernatant was discarded. The final Mt pellet was depolymerized on ice in ~2 ml PME buffer for 20 minutes, then clarified at 70k × g, 4°C, 10 min, in a TLA100.3 rotor (Beckman, Palo Alto, CA). Aliquots were snap-frozen in liquid nitrogen and stored at ~80°C. Yield was 6–8 mg of highly purified tubulin per 100 ml of dejelled eggs. Purification using only a 1 M glutamate-cycle on the 2-cycled tubulin without DEAE chromatography was tested, but the resulting tubulin was not as pure and was therefore not utilized.

Mt Sedimentation Assay

Sedimentation of Mts was performed to determine the steady-state critical tubulin concentration and dead-tubulin fraction according to Johnson and Borisly [1977] and Detrich and Wilson [1983] as follows. Urchin egg tubulin in PME buffer was quickly defrosted, then allowed to polymerize at 37°C for 15 min. The Mts were sedimented at 70k × g, 37°C, 10 min, TLA100.3 rotor, and the supernatant was discarded. The Mt pellet was resuspended into the desired buffer and allowed to depolymerize on ice for 20 min. In some experiments, tubulin in PME buffer was defrosted and used directly in the assays. Various dilutions of the tubulin were made into 1.5 ml polypropylene microfuge tubes. The samples were then incubated at 25°C for 1 h, and sedimented as above but at 25°C. Aliquots of the supernatant were withdrawn, and the protein concentrations were measured according to Bradford [1976] using reagents from Pierce (Rockford, IL) or Bio-rad Laboratories (Richmond, CA) with bovine serum albumin as the protein standard. The sedimentation conditions were sufficient to sediment polymer since samples centrifuged for times ranging from 5 to 30 min showed identical results. Likewise, samples incubated on ice and sedimented at 4°C did not show a decrease in tubulin concentration in the supernatant, suggesting that tubulin dimers did not sediment. All calculations assumed that the urchin tubulin Mr = 100 kD.

Analysis of Dynamic Instability of Individual Mts by VE-DIC Microscopy

VE-DIC microscopy was used to directly visualize individual Mts in real time [Salmon et al., 1989; Walker et al., 1988]. Kinetic rate constants and transition fre-
quencies were obtained by measuring the length of the visualized Mts vs. time by the same techniques that have been previously applied to purified brain tubulin [Walker et al., 1988]. Purified tubulin samples were prepared by cycling urchin tubulin into the desired buffer just before use as described above, or directly from storage at −80°C. A volume of tubulin, 1/10 or 1/5 vol isolated Tetrahymena axonemes (see below), and buffer were combined in a small polyethylene tube on ice, then 6 μl of the sample was applied to a glass slide (ethanol wiped), covered with a detergent/water/ethanol sonication-cleaned #0 glass 22 × 22 mm coverslip, and sealed in valap (1:1:1 vaseline/lanolin/paraffin). Egg cytoplasmic extract samples were prepared by adding 10% vol/vol isolated sperm axonemes in HME0 buffer (see below) to the defrosted extract, plus GTP/cytchalasin B at 1/50 vol/vol to a final concentration of 1 mM/10 μM. Extract filtrate/tubulin samples were prepared by resuspension of sedimented Mts directly into filtrate. The tubulin was further diluted during assay with filtrate. The slides were prepared as above. The samples were then placed on the microscope stage at room temperature (23–25°C) and examined by DIC light microscopy using the microscope described by Walker et al. [1990] with video-enhanced contrast described by Salmon et al. [1989]. The analysis was performed according to Walker et al. [1988], which assumed that the Mt elongation velocity can be described by a simple bimolecular rate equation (see Table II legend).

**Other Methods**

Isolated axonemes used in the purified tubulin experiments were prepared from Tetrahymena thermophila, strain SB255 (nuclease deficient), by a modification of Thompson et al. [1974], Witman et al. [1978], and Telzer and Haimo [1981] as detailed in Simon and Salmon [1990]. The S. purpuratus sperm axonemes used in the egg cytoplasmic extracts were prepared according to Bell et al. [1982] as described by Walker et al. [1988]. The axonemes were stored at −20°C in 50% glycerol. Before use, axonemes were diluted >15-fold with the assay buffer, sedimented at 7k × g for min at 4°C, the supernatant was discarded, and the axonemes were resuspended back to the original volume with the assay buffer.

The Mt assay buffers were as follows: PMEO (0.1 M Pipes, 5 mM MgSO₄, 2 mM EGTA, 1 mM GTP, pH 6.9 at r.t.), PME1 (PME0 at 1 μM free calcium; includes 1.24 mM CaCl₂), PME pH 7.2 (same as PMEO but at pH 7.2), HME0 (0.1 M Hepes, 5 mM MgSO₄, 2 mM EGTA, 1 mM GTP, pH 7.2 at r.t.), and HME1 (HME0 at 1 μM free calcium; includes 1.70 mM CaCl₂). Free calcium concentrations were calculated according to Robertson and Potter [1984], which assumed identical association constants for ATP and GTP [see Martell and Smith, 1974].

SDS-PAGE was performed according to Laemmli [1970], and stained with 0.1% Coomassie Blue R-250 [Weber and Osborn, 1969] and destained in 10% acetic acid, or silver-stained [Merril et al., 1980; Morrissey, 1981].

**RESULTS**

**Dynamic Instability in Cytoplasmic Extracts**

Cytoplasmic extracts were prepared as described by Glikman and Salmon (in preparation) from unfertilized S. purpuratus sea urchin eggs using an isosmotic Hepes/glycine lysis buffer (HEMG: 180 mM Hepes, pH 7.35, 10 mM MgCl₂, 10 mM EGTA, 0.5 M glycine) and centrifugation to clarify the extracts of membrane vesicles and yolk granules. Although the extract was prepared from unfertilized eggs, it can be considered a pH-activated cytoplasm since the HEMG lysis buffer raises the pH of the unfertilized egg cytosol from about 6.9 to the measured value of 7.25, the pH of the fertilized eggs [Schatten et al., 1985; Dube et al., 1985]. The Ca²⁺ concentration was also clamped to less than 10 nM by the EGTA in the HEMG lysis buffer. The HEMG lysis buffer dilutes the cytosol concentration about 30–50% by volume in our clarified extracts (Glikman and Salmon, in preparation). The endogenous tubulin concentration in the clarified extracts was 13.7 μM (SD = 1.9; n = 5; 2 extracts), and the total protein concentration was 24.3 mg/ml. In our video assembly assays, the tubulin concentration was diluted to 12.1 μM by the addition of GTP necessary for tubulin assembly, isolated axoneme fragments to nucleate Mt growth, and cytchalasin B to prevent actin filament assembly.

When extracts warmed to 25°C were viewed with the VE-DIC microscopy assay of Walker et al. [1988], Mt were seen to grow only from the plus ends of the axonemes as reported earlier for Mt assembly in cytoplasmic extracts of Xenopus by Gard and Kirschner [1987] and sea urchin egg extracts by Glikman and Salmon (in preparation). Most growth occurred from axonemes while only a few scattered free Mts were seen. Over several minutes, the net length of the Mts increased, with many Mts achieving >30 μm lengths (field of view).

Analysis of the polymerization of individual Mts showed that they exhibited dynamic instability (Fig. 1; Table I) with the elongation velocities, and frequent catastrophes and rescues typical of interphase cytoplasm [Cassimeris et al., 1988; Sammak and Borisy, 1988; Schulze and Kirschner, 1988; Belmont et al., 1990] (Glikman and Salmon, in preparation). Growth occurred with an average elongation velocity of 7.8 μm/
min and a mean elongation duration of 1.3 min before catastrophe into the rapid shortening phase. The Mts rapidly shortened at 13 μm/min on average and they persisted in rapid shortening for only about 0.5 min before rescue back into the elongation phase. Although catastrophe during elongation was frequent, an individual Mt had a net increase in length of (7.8 μm/min × 1.3 min) − (13 μm/min × 0.5 min) = 3.64 μm in 1.8 min because of the relatively high frequency of rescue. This signifies that the average extract Mt will display a continual net elongation. Elongation at 7.8 μm/min velocity at 12.1 μM tubulin concentration corresponds to a growth rate of 0.64 μm/min/μM tubulin.

**Dynamic Instability in Standard Brain Mt Reassembly Buffer**

It was important to obtain sea urchin egg tubulin free of MAPs. This was accomplished by first preparing Mt protein from unfertilized S. purpuratus eggs according to Suprenant and Marsh [1987] which employed 8% DMSO and pH 7.3 Pipes buffer to promote Mt assembly at 25°C. This 2-cycled tubulin was further purified by DEAE chromatography according to Keller and Rebhun [1982]. An SDS-PAGE of the DEAE step is shown in Figure 2. After desalting the tubulin by gel filtration, sodium glutamate (1 M) was added to promote assembly and to remove trace contaminating Mt MAPs [Hamel and Lin, 1981; Voter and Erickson, 1984]. The effectiveness of the latter step is illustrated in Figure 2. This is an SDS-PAGE of an urchin egg tubulin purification in which the DEAE column was overloaded with protein which resulted in an unusually high level of protein contaminants (lane C). After desalting the tubulin (lane D), which did not add to the purification, the glutamate-cycle was performed. This step removed all silver-stained detectable contaminants (lane E).

Initially the assembly of the highly purified egg tubulin was analyzed at 25°C in a Pipes buffer commonly used for the assembly of brain tubulin (PME0: 0.1 M Pipes, 5 mM MgSO₄, 2 mM EGTA, 1 mM GTP, pH 6.9). Typically, 1 mM magnesium is used in PME buffer for brain Mt reassembly [Walker et al., 1988; O'Brien et
al., 1990). We used 5 mM MgSO₄ in this study because this higher concentration is closer to urchin egg physiological levels [Rothschild and Barnes, 1953] and because higher magnesium concentrations make reassembled brain Mts more dynamic by increasing the elongation and rapid shortening velocities [O'Brien et al., 1990]. The Mt kinetic parameters were measured by the VE-DIC microscopy assay. The range of tubulin concentrations tested was limited to the minimum needed to nucleate any Mts from the isolated axoneme seeds, and to the maximum before there was significant self-assembly. As with brain tubulin [Walker et al., 1988], urchin egg tubulin Mts were seen to elongate from both the plus and minus ends of axonomes, but only kinetic data for the plus ends were obtained. Plus ends were discriminated from minus ends by their higher growth velocity and increased ability to nucleate from the axoneme ends. The plus-end polarity was directly confirmed in some instances by their elongation from the frayed, distal axoneme ends [Borisy and Bergen, 1982], which occurred infrequently in the Tetrahymena axoneme preparations. Plus-end Mt elongation velocity vs. tubulin concentration is shown in Figure 3. The kinetic rate constants obtained from Figure 3 are tabulated in Table II which assumed that the Mt elongation velocity can be described by a simple bimolecular rate equation (see Table II legend) [Walker et al., 1988]. Plus-end Mts assembled from urchin egg tubulin in PME0 buffer display similar elongation velocities as purified brain tubulin in a similar buffer [O'Brien et al., 1990], as compared in Table III, but about half that obtained in the urchin egg extract (3.5 vs. 7.8 μm/min) when normalized to the same tubulin concentrations (Tables I, II).

The assembly of urchin egg tubulin in PME buffer was much more stable than in the cytoplasmic extracts (Fig. 1) and more stable than the assembly of pure brain tubulin in PME buffers (Table III) [O'Brien et al., 1990]. The frequency of catastrophe for egg tubulin in PME buffer was extraordinarily low, even at very low tubulin concentrations and slow growth rates. At 1 μm/min growth velocity, a catastrophe in PME0 occurred on average every 5.6 min (Table IV), in contrast to every 1.3 min in the cytoplasmic extracts (Table I) and 2.8 min for brain tubulin assembly (Table III) [O'Brien et al., 1990]. Since catastrophe frequency is inversely sensitive to elongation velocity for brain tubulin [Walker et al., 1988; O'Brien et al., 1990] and urchin egg tubulin (data not shown), catastrophe frequencies for urchin egg tubulin in PME0 buffer could be an order of magnitude less frequent at 7.8 μm/min.
TABLE II. Purified Sea Urchin Egg Mt Kinetic Rate Constants

<table>
<thead>
<tr>
<th>Graphical parameter</th>
<th>Kinetic parameter</th>
<th>PME0</th>
<th>PME pH 7.2</th>
<th>HME0</th>
<th>HME1</th>
<th>PME1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope (µm/min/µM)</td>
<td>k_{eff}</td>
<td>0.325</td>
<td>0.603</td>
<td>0.086</td>
<td>0.092</td>
<td>ND²</td>
</tr>
<tr>
<td>X-intercept (µM)</td>
<td>[critical]elong</td>
<td>1.4</td>
<td>1.8</td>
<td>5.8</td>
<td>4.5</td>
<td>ND</td>
</tr>
<tr>
<td>Y-intercept (µm/min)</td>
<td>k_{-1}eff</td>
<td>-0.45</td>
<td>-1.10</td>
<td>-0.50</td>
<td>-0.42</td>
<td>ND</td>
</tr>
<tr>
<td>X-intercept (µM)²</td>
<td>SSₘₐₓ</td>
<td>1.7</td>
<td>1.5</td>
<td>18.7</td>
<td>17.9</td>
<td>1.2</td>
</tr>
</tbody>
</table>

¹These values were derived by linear regression from the video graphs assuming that the Mt elongation velocity follows the following bimolecular rate equation: V_{eff} = k_{eff}[s] + k_{-1}eff, where V_{eff} = plus-ended elongation velocity, k_{eff} = the bimolecular association rate constant, [s] = free tubulin concentration, and k_{-1}eff = dissociation rate constant.
²Buffer conditions are 0.1 M Pipes (PMEx) or Hepes (HEEx) with 5 mM MgSO₄; 2 mM EGTA, and 1 mM GTP @ 25°C. HME1 and PME1 have CaCl₂ added to give 1 µM free calcium. PME0 and PME1 are at pH 6.9, while HME0, HME1, and PME pH 7.2 are at pH 7.2.
³The steady-state critical concentrations (SSₘₐₓ) were derived from the sedimentation graphs.
⁴ND = not determined.

TABLE III. Dynamic Instability Properties of Purified Sea Urchin Egg Vs. Brain Mt Plus Ends Under Similar Buffer Conditions

<table>
<thead>
<tr>
<th>Kinetic parameter</th>
<th>Sea urchin egg (25°C)</th>
<th>Porcine brain (37°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>k_{eff} (µM/min/µM)</td>
<td>0.33</td>
<td>0.33</td>
</tr>
<tr>
<td>[critical]elong (µM)</td>
<td>1.4</td>
<td>2.7</td>
</tr>
<tr>
<td>k_{-1}eff (µM/min)</td>
<td>-0.45</td>
<td>-0.89</td>
</tr>
<tr>
<td>V₃₀ (µM/min)</td>
<td>-5.4</td>
<td>-84.2</td>
</tr>
<tr>
<td>[Tu]ₘₐₓ (µM/min)</td>
<td>4.7</td>
<td>5.7</td>
</tr>
<tr>
<td>tₙ (min)</td>
<td>5.6</td>
<td>2.8</td>
</tr>
<tr>
<td>tₙ (min)</td>
<td>0.4</td>
<td>-0.8</td>
</tr>
</tbody>
</table>

¹The egg kinetic parameter terminology is identical to Tables II and IV.
²The urchin egg tubulin parameters are in PME0 buffer.
³Purified porcine brain tubulin properties are taken from O'Brien et al. [1990] and were assayed in 0.1 M Pipes, 6 mM MgSO₄, 2 mM EGTA, pH 6.9, which is identical to the PME0 buffer except for the inclusion of 6 vs. 5 mM Mg²⁺.
⁴[Tu]ₘₐₓ = concentration of tubulin required to obtain an elongation velocity of 1 µm/min.
⁵tₙ = inverse catastrophe (tₙ) or rescue (tₙ) frequency (see Table IV) at the tubulin concentration which supports an elongation velocity of 1 µm/min.

The Mts assembled from urchin egg tubulin in PME0 buffer were also more stable than Mts in egg cytoplasmic extracts and assembled from brain tubulin because of a higher rescue frequency (tₙ = 0.4 min vs. 0.5 and 0.8 min, respectively; Tables IV, I, and III) and slower rapid shortening velocities (5 µm/min vs. 13 and 84 µm/min, respectively). The net Mt polymer disassembled during a rapid shortening event in PME buffer (tₙ × Vₙₚ) was only ½ the amount lost in the cytoplasmic extracts, and much less than that for brain tubulin in PME0 buffer. The lower frequency of catastrophe, lower velocity of rapid shortening, and higher rescue frequency all contribute to the unexpected stability of egg tubulin assembly in PME0 buffer.

The stability of egg tubulin assembly in PME0 buffer reflected in the VE-DIC microscopy assay data was also demonstrated by a low steady-state critical tubulin concentration (SSₘₐₓ) as measured by a sedimentation assay (see Materials and Methods). The SSₘₐₓ for urchin egg tubulin in PME0 buffer at 5 mM magnesium was 1.7 µM (Fig. 4; Table II). The tubulin concentration in cytoplasmic extracts was 7 times higher than this value, indicating that massive Mt self-assembly would occur in PME0 at this concentration. For a comparison, brain tubulin SSₘₐₓ is reported at ~7 µM [Walker et al., 1988] in PME buffer at 1 mM magnesium and 37°C, while urchin egg tubulin under identical conditions was 1.5 µM (data not shown).

Variations in Reassembly Buffer Composition

We next tested the possibility that one of the components of the PME reassembly buffer, other than magnesium, had a profound stabilizing effect on the assembly of egg tubulin. Three buffer factors known to promote lability of Mts were examined: higher pH [Regula et al., 1981; Hays and Salmon, 1986], changing from Pipes to Hepes [Olmsted and Borisy, 1975; Himes et al.,...
Fig. 4. Steady-state critical assembly concentration of urchin tubulin in Pipes (PME0, PME1) vs. Hepses (HME0, HME1) buffers with 1 μM free calcium (PME1, HME1) or without calcium. Urrchin egg tubulin samples at various concentrations were allowed to polymerize at 25°C for 1 h, and then were sedimented at 70k × g for 10 min (see Materials and Methods). Samples of the supernatant were analyzed for protein concentration (B, C) which were used to calculate the Mt polymer concentrations (A). The X-intercept in A, and the steady tubulin concentration post 5 μM in B and 20 μM in C represent the steady-state concentration. The non-linearity below ~5 μM in B was due to nucleation effects since no exogenous seeds were added, and those points were not curve fit in A. The slope in A represents the fraction of tubulin competent to assemble into Mt and HME1 (100 ± 5%). Pip values (squares) had an approximately 10-fold lower SSS crit than Hepses buffers (triangles) (see Table I). Calcium had no effect as shown by the overlapping of points in B and C (open and closed symbols). Interestingly, the non-linearity due to nucleation at tubulin concentrations ~5 μM observed for Pipes buffers in B was not observed for Hepses (C).

1979; Waxman et al., 1981], and 1 μM Ca²⁺ [Kiehart, 1981; Salmon and Segall, 1980].

Higher pH. When the pH of PME buffer was raised from 6.9 to 7.2 (the pH of activated egg cytosol), Mt elongation was faster (0.6 vs. 0.3 μm/min/μM tubulin), the frequency of catastrophe was lower (1/7.6 vs. 1/5.6 min⁻¹), and the rate of rapid shortening and frequency of rescue did not change significantly (Fig. 5, 6; Tables II, IV). The SSS crit remained at ~2 μM, the same as at pH 6.9 (data not shown). By raising the pH of PME buffer, Mt growth rates became similar to those in the cytoplasmic extracts (an estimated 6.1 μm/min for 12 μM tubulin; Fig. 5 and Tables I, II), but MtS were still much more stable than in the extracts (Tables I, IV).

Changing from Pipes to Hepses. Switching from 100 mM Pipes to 100 mM Hepses in the Mt reassembly buffer at pH 7.2 (HME0 buffer) had a profound effect on pure egg tubulin assembly. Hepses was the buffer chosen to buffer pH in the preparation of cytoplasmic extracts because its pKa is near the cytosol pH of activated eggs. After dilution by the cytosol, the Hepses concentration was in the range of 50 to 100 mM in our cytoplasmic extracts.

In HME0 buffer, Mt growth required much higher tubulin concentrations and MtS were much less stable than in PME buffer (Figs. 1, 3). Video analysis showed that nucleation were infrequent below about 12 μM tubulin and Mt self-assembly did not occur below 20 μM tubulin. Mt elongation in HME buffer occurred at only 0.086 μm/min/μM tubulin, about 7 times slower than in PME buffer at pH 7.2. In addition, the apparent critical tubulin concentration for elongation was 5.8 μM, about
3 times higher than in PME buffer (Fig. 3; Table II). At the 12 µM tubulin concentration of the clarified extracts, Mts in HME buffer would elongate at (12–5.8) × 0.086 = 0.5 µm/min velocity, about 15 times slower than in the cytoplasmic extracts. As seen in Figure 1 and Table IV, the duration of elongation before a catastrophe in HME0 buffer was similar to that in PME buffer at 1 µm/min growth velocity. In contrast, the frequency of rescue and the velocity of rapid shortening were very different. No rescues were seen in HME buffer and the rapid shortening velocity, 15 µm/min, was almost 3 times faster than in PME buffer and similar to the rate in the cytoplasmic extracts (Fig. 6; Table I).

The lack of rescue as well as the slow growth and fast rapid-shortening rates in HME buffer help explain why the SS_{crit} was about 18 µM in HME buffer compared to 2 µM in PME buffer (Table II; Fig. 4). The slopes of the fitted lines were almost identical and equal to one indicating that 100% (±5%) of the tubulin was active.

1 µM Ca^{2+}. Although the concentration of calcium in the extracts was held to 10 nM or less by EGTA, the effect of 1 µM Ca^{2+} was tested on urchin egg tubulin assembly in both the PME and HME buffers to see if

**Assembly in Filtered Cytosol**

Since Mtx dynamics in the sea urchin cytoplasmic extracts could not be mimicked by purified urchin egg tubulin in either the PME or HME buffers tested above, the assembly of purified egg tubulin was tested in the native low molecular weight buffer of the extracts. Filtrates were obtained by ultrafiltration of active egg extracts (Table I) through 30 kD MWCO centrificon devices. These filtrates contained only proteins much less than 30 kD as seen in silver-stained SDS-PAGE (data not shown). The total protein concentration of the filtrates was 0.24 mg/ml, much less than the 24 mg/ml total protein concentration characteristic of urchin egg extracts.

Purified egg tubulin was added back to these ultrafiltrates to a final concentration in the video assay mixtures of 11.9 µM, the concentration of tubulin in unfiltered cytoplasmic extracts. In the extract ultrafiltrate at this tubulin concentration, Mtx assembled off both the plus and minus ends of axonemes, indicating no inhibition of minus-end growth as occurs in the unfiltered extract. At slightly higher tubulin concentration there was
**TABLE V. Mt Kinetics in Egg Cytoplasmic Ultrafiltrate**

<table>
<thead>
<tr>
<th>Kinetic parameter&lt;sup&gt;*&lt;br&gt;(μm/min)</th>
<th>Value&lt;br&gt;(SD = 0.25; n = 33)</th>
<th>Value&lt;br&gt;(SD = 2.82; n = 5)</th>
<th>Observation time = 130 min</th>
<th>Observation time = 4 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_e$</td>
<td>1.2</td>
<td>11.8</td>
<td>25.9</td>
<td>0.9</td>
</tr>
</tbody>
</table>

*Purified egg tubulin was cycled into cytoplasmic filtrates, prepared by ultrafiltration of concentrated cytoplasmic extracts of unfertilized *S. purpuratus* sea urchin eggs (see Materials and Methods). The MW cut-off of the filtrate was $<30$ kDa and had a protein concentration of 0.24 mg/ml. The final tubulin concentration was adjusted to 11.9 μM with filtrate, and plus-ended MTs were analyzed at 25°C. Minus-ended MTs were also observed but were not quantitated.

$V_e$ = average elongation ($V_e$) and rapid shortening ($V_{rs}$) velocity. $t_e$ = inverse catastrophe ($t_e$) and rescue ($t_r$) frequency (see Table IV).

massive self-assembly, while at a slightly lower concentration no assembly occurred.

Mts also grew slowly, but were extraordinarily stable in the filtered extracts (Table V). At 11.9 μM tubulin, the elongation velocity was 1.2 μm/min, about 7 times slower than in the unfiltered extract (Table I). Catastrophe was very infrequent (1/26 min elongation) in comparison to the extract (1/1.3 min elongation). The rate of rapid shortening (12 μm/min) was similar to the values measured for unfiltered extract and HME buffer. Unlike in the HME buffer, rescue was sufficiently frequent in the extract ultrafiltrates so that a Mt always switched back to elongation before shortening to completion. This produced net Mt elongation as in the cytoplasmic extracts, and PME buffers.

**DISCUSSION**

Mt dynamics in the *S. purpuratus* egg cytoplasmic extracts are typical of interphase Mts in living non-neural cells and interphase-like extracts. They are characterized mainly by high elongation velocities with high catastrophe frequencies, often with frequent rescues [Cassimeris et al., 1988; Belmont et al., 1990] (Gilkosman and Salmon, in preparation). In contrast, the vast majority of Mts in neural cells are virtually static [Okabe and Hirokawa, 1988; Seitz-Tutter et al., 1988]. If the intrinsic properties of the tubulin dictates the in vivo Mt dynamics, then purified brain tubulin should be less dynamic than urchin egg tubulin. Surprisingly, the exact opposite result was obtained. A comparison between Mt assembled in Pipes buffer from purified sea urchin egg tubulin and porcine brain tubulin [O’Brien et al., 1990] is shown in Table III. Purified porcine brain tubulin is the only other tubulin in which the dynamic instability properties of individual Mts have been measured [Walker et al., 1988; O’Brien et al., 1990]. The results indicate that the urchin egg tubulin assembles at a slightly lower concentration, shortens more slowly, and has a lower catastrophe frequency with higher rescue compared to the brain tubulin. These differences would be even more accentuated if the measurements were normalized to the same temperature. For example, the urchin egg tubulin assembles much better at 37°C (not shown), while brain tubulin is less stable at 25°C [Walker et al., 1989]. The net effect is that the urchin tubulin is more stable than brain tubulin under these buffer conditions. The surprising conclusion is that Mts assembled from purified urchin tubulin are less dynamic than for brain tubulin. Therefore, the endogenous brain and sea urchin egg tubulins have inverse assembly characteristics in vitro and in vivo.

The above conclusion depends on the assumption that the urchin egg tubulin we purified is unmodified from its state in the cytoplasm. In fact, the extracts and tubulin were prepared from identical starting material (unfertilized *S. purpuratus* sea urchin eggs), and in both cases the initial egg homogenates were pH activated by clamping the pH to 7.3. The relative stability of the urchin egg tubulin compared to the extracts and brain tubulin was not due to tubulin modification by our purification steps. The urchin egg tubulin protocol described here employed 8% DMSO and elevated pH (pH 7.3) to initiate polymerization of Mts from crude homogenates, followed by DEAE chromatography and sodium glutamate cycling. The advantage of the initial DMSO/pH 7.3 steps is that tubulin and cosedimenting MAPs can be obtained simultaneously [discussed by Suprenant and Marsh, 1987; Suprenant, 1989]. This can potentially allow the concerted purification of tubulin and MAPs from the egg. The use of sodium glutamate serves two purposes. First, it assures reproducible purity of the tubulin (see Fig. 1) by salting-off any trace MAPs [Voter and Erickson, 1984]. Second, it increases the tubulin yields during cycling by reducing the steady-state critical concentration, thereby minimizing loss in the supernatant [Hamel and Lin, 1981]. It can be argued that DMSO and/or sodium glutamate could irreversibly lower the critical concentration of the tubulin. However, unfertilized sea urchin tubulin purified by other methods had similar steady-state concentrations. Detrich and Wilson [1983] prepared egg extracts at pH 6.82 and directly employed DEAE chromatography, followed by two cycles of polymerization/depolymerization. Suprenant and Rebhan [1983] employed glycerol in the initial egg homogenization followed by ammonium sulfate precipitation and DEAE chromatography. Suprenant and Marsh [1987] used homogenization buffer at pH 7.3 and 8% DMSO to induce polymerization, followed by 2 to 3 cycles of polymerization/depolymerization. In all cases, the steady-state critical concentrations ranged from 0.11 to 0.3 mg/ml between 24 and 37 °C in PME buffer,
similar to our results which were 0.2 and 0.15 mg/ml at 25 and 37°C, respectively. In addition, Suprenant [1989] purified surf clam tubulin according to the method of Suprenant and Marsh [1987], and determined a critical concentration in PME buffer of 1.65 mg/ml at 22°C, which was ~6 times higher than the sea urchin egg tubulin purified by the same methods. This suggests that at least the DMSO and elevated pH cannot account for the low critical concentrations found in the urchin egg tubulin. The finding that DMSO and glutamate cycling are apparently reversible are not surprising since their mechanism of action is probably not stoichiometric, but rather due to changing the solvent properties to favor polymerization [Lee and Timasheff, 1977; Suzaki et al., 1978].

The pH of unfertilized sea urchin eggs was found to be 6.9 and 7.0 for *L. pictus* and *S. purpuratus*, respectively [Dube et al., 1985]. After fertilization, the pH rises to approximately 7.2 within a few minutes [Dube et al., 1985; Schatten et al., 1985]. When the pH shift is blocked, Mts do not assemble in the eggs. Likewise, if the pH is artificially increased to post-fertilization levels, Mts assemble suggesting that the pH rise itself is sufficient for Mt assembly [Schatten et al., 1985]. Mts assembled from purified urchin egg tubulin displayed a higher elongation velocity at pH 7.2 vs. 6.9. At the tubulin concentration in the egg extracts (12 µM), the extrapolated velocity is doubled at the higher pH. This suggests that pH may have a direct role in modulating *Mt* dynamics in vivo. This is in contrast to the bulk assembly properties of brain tubulin which favor lower pH [Olmsted and Borisy, 1975; Himes et al., 1979; Regula et al., 1981].

The assembly of purified urchin egg tubulin was assayed in two different pH buffers: Pipes and Hapes. Originally, these buffers were chosen simply because of their optimal buffering capacity at the unfertilized and activated cell pHs of 6.9 and 7.2, respectively [Dube et al., 1985; Schatten et al., 1985]. Subsequently, it was realized that the buffer itself had a profound affect on the Mt properties regardless of pH. The main effect of Pipes was to greatly stabilize Mt assembly relative to Hapes. Specifically, the stability in Pipes is expressed as a 4-fold increase in the bimolecular association constant (slope), a 4-fold lower critical concentration, a high rate of rescue relative to Hapes, a 10-fold lower SS_{crit}, and a 3-fold lower rapid-shortening velocity. Therefore, plus-ended Mt elongation is always faster in Pipes at a given tubulin concentration, and Mts will tend to grow much longer. Although the mechanism is unknown, it is well established that Pipes and Hapes can stimulate Mt assembly, as can the commonly used Mes buffer [Olmsted and Borisy, 1975; Himes et al., 1979; Waxman et al., 1981].

Pipes has been shown to be a better Mt assembly stimulator than either Hapes or Mes [Himes et al., 1979; Waxman et al., 1981]. All three buffers are sulfonates, and in fact simple methyl, ethyl, propyl, and butyl sulfonates can stimulate assembly to different extents, the 1 and 2 carbon species being most effective [Himes et al., 1979]. Other buffers such as Tris, phosphate and imidazole show no assembly stimulation [Waxman et al., 1981]. Walker et al. [1988] and O'Brien et al. [1990] found that catastrophe frequency for brain tubulin decreases at high tubulin concentration, as it does for urchin egg tubulin. However, the catastrophe frequency for purified urchin egg tubulin was much higher in Hapes than in Pipes buffer relative to tubulin concentration. This indicates that catastrophe frequency is not strictly tubulin concentration dependent, but also depends on the hydrogen ion buffer employed.

Calcium concentration at \( <10 \text{ nM} \) vs. 1 µM had no measurable affect on the SS_{crit} or on Mt dynamic instability. The lack of calcium effect at this level is in agreement with Keller et al. [1982] with *S. purpuratus* purified spindle tubulin. They found in studies of Mt self-assembly that only nucleation was sensitive to micromolar calcium, and more so at lower temperatures (18°C). However, at higher calcium concentrations (>10 µM) it is well established that polymerization of purified tubulin is calcium sensitive [Olmsted and Borisy, 1975; Berkowitz and Wolff, 1981; Gal et al., 1988]. The relatively high free magnesium concentrations in our buffers compared to standard brain buffers could have inhibited or masked subtle calcium effects by divalent cation substitution. Since there was no Mt rescue in Hapes buffer, any affect of calcium on the rescue transition would be missed. It is known that Mts in vivo and in isolated mitotic spindles are sensitive to calcium concentrations at the micromolar level [Salmon and Segall, 1980; Kiehart, 1981]. This calcium sensitivity may involve factors such as a direct modulation by MAPs or via a calcium-sensitive cascade involving phosphorylation of MAPs and/or tubulin itself [Dinsmore and Sloboda, 1989].

In Table VI, a comparison is made between Mt dynamics in unfiltered sea urchin egg extracts, and purified sea urchin egg tubulin in cytoplasmic ultrafiltrates, and Pipes and Hapes buffer calculated at the egg extract tubulin concentration (12.1 µM). If ambient cytosolic conditions were mimicked by Pipes buffer at pH 7.2, purified urchin egg tubulin would have an extrapolated velocity of 6.1 µm/min. This velocity is similar to that in the extracts, however, there would be a problem of massive self-assembly (SS_{crit} = 1.5 µM), very low catastrophe frequency, and Mt rapid shortening that is ~3 times too slow in this buffer. If the cytosol were more Hapes-like (HME0), then the elongation velocity would be much too slow at 0.5 µm/min, and the time between catastrophes would be much too long (~8 min). How-
TABLE VI. Urchin Egg Mt Dynamic Instability in Cytoplasmic Extracts Vs. That in Cytoplasmic Filtrate and Buffers

<table>
<thead>
<tr>
<th>Kinetic parametera</th>
<th>Cytoplasmic</th>
<th>Filtratec</th>
<th>Buffersd</th>
<th>PME pH 7.2</th>
<th>HME0</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_c$ (μm/min)</td>
<td>7.8</td>
<td>1.2</td>
<td>6.1</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>$t_c$ (min)</td>
<td>1.3</td>
<td>25.9</td>
<td>&gt;8</td>
<td>-8</td>
<td></td>
</tr>
<tr>
<td>$V_{rs}$ (μm/min)</td>
<td>13.0</td>
<td>11.8</td>
<td>5.2</td>
<td>15.2</td>
<td></td>
</tr>
<tr>
<td>$t_{rs}$ (min)</td>
<td>0.5</td>
<td>0.9</td>
<td>0.5</td>
<td>NO*</td>
<td></td>
</tr>
</tbody>
</table>

aThe kinetic parameter terminology is the same as Tables II and IV. Note that $t_c$ and $t_{rs}$ are the average times between a catastrophe during elongation and rescue during rapid shortening, respectively.
bConcentrated egg cytoplasmic extract values were taken from Table I (endogenous tubulin = 12.1 μM). cPure egg tubulin was cycled into ultrafiltrates of the cytoplasmic extracts (tubulin = 11.9 μM; data taken from Table V). dBuffers parameters were extrapolated from values in Tables II and IV assuming a tubulin concentration of 12.1 μM (urchin egg extract assay concentration). eNO* = not observed.

However, the Heps buffer mimics the in vivo characteristics in the rate of rapid shortening and the lack of self-assembly at this concentration. Therefore, the assembly of purified tubulin in either Pipes or Heps buffer cannot mimic the extract results. This comparison also shows that Heps, rather than Pipes, is the buffer of choice for in vitro reconstitution experiments.

Comparing the dynamic instability of Mts in ultrafiltrates to unfiltered extracts shows that most of the parameters of dynamic instability are regulated by factors which do not pass through the filter. These include factors which (Table VI): 1) stimulate plus-end Mt elongation velocity by 6.5-fold without concurrent self-assembly; 2) increase catastrophe frequency by 20-fold; and 3) completely block minus-end assembly. MAPs from brain, MAP2 and tau, have been shown to greatly stimulate Mt elongation velocity (Pryer et al., in preparation), however, it simultaneously suppresses catastrophe and also lowers the $SS_{crit}$ which leads to massive self-assembly under our conditions. Although XMAP, a 215 kD protein from Xenopus eggs, also stimulates elongation velocity [Gard and Kirschner, 1987], it is not known how the other dynamic instability properties are changed. Presently, there are no known cytoplasmic catastrophe factors or minus-end blockers. Finding these factors is critical for understanding how Mt assembly is regulated in the cell.

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