Chapter 5

Mitotic Spindles Isolated from Sea Urchin Eggs with EGTA Lysis Buffers

E. D. SALMON

Department of Zoology
University of North Carolina
Chapel Hill, North Carolina

I. Introduction ........................................... 70
II. In Vivo Spindle Characteristics ....................... 73
   A. Definition of Spindle Parts .......................... 73
   B. Spindle-Fiber Microtubules ......................... 75
   C. Other Components .................................. 77
   D. Microtubule Lability ............................... 79
   E. Egg Cortex ........................................ 79
III. Approaches to in Vitro Spindle Models ............... 81
IV. Evolution of EGTA Lysis Buffer ....................... 82
   A. Historical Considerations ........................... 82
   B. EGTA Lysis Buffer .................................. 83
   C. Physiological Considerations ...................... 83
   D. Species Specificity ................................ 85
   E. Other Cell Types .................................. 85
V. Procedures for Isolating Spindles ..................... 86
   A. General Considerations ............................. 86
   B. Methods for L. variegatus ......................... 90
   C. Methods for S. droebachiensis ..................... 93
VI. Characteristics of Spindles Isolated in EGTA Lysis Buffers 94
   A. Structural Characteristics .......................... 94
   B. Biochemical Characteristics ....................... 95
   C. Physiological Characteristics ..................... 97
VII. Future Considerations ................................ 100
    References ......................................... 102
I. Introduction

This chapter describes methods used in our laboratory to isolate mitotic spindles from dividing sea urchin eggs. An obvious goal is to obtain a preparation that has a well-defined composition, that can be stored, and that can then be reactivated under controlled conditions to produce lifelike assembly characteristics and chromosome transport (Inoué, 1964; Inoué and Sato, 1967; Inoué et al., 1975; Nicklas, 1971, 1975, 1977; Salmon, 1975, 1976).

The mitotic apparatus is a complex organelle, and an ideal isolated model has not yet been achieved. The spindle-fiber microtubules are labile and will generally depolymerize within several minutes if cells are lysed into normal culture media. Several spindle-isolation techniques have been developed that use glycols or purified brain microtubule protein (MTP) to stabilize the spindle microtubules on cell lysis. However, these procedures significantly alter the native assembly characteristics of the spindle microtubules, hence no activated chromosome movements have been obtained (reviewed by McIntosh, 1977; Salmon et al., 1980). Significant progress toward a functional isolated model is being made, in part by recognizing that spindle-fiber microtubules are sensitive to depolymerization by micromolar concentrations of calcium ions and that calcium ions are normally sequestered below $10^{-7}$ M within the spindle microenvironment (Inoué and Kiehart, 1978; Kiehart, 1979; Kiehart and Inoué, 1976; Salmon and Jenkins, 1977; Salmon and Segall, 1980; Weisenberg, 1978).

We have found that spindles with relatively stable microtubules can be isolated from dividing sea urchin eggs using simple EGTA lysis buffers. The morphology and birefringent retardation (BR) of these isolated spindles are similar to spindles in living cells (Figs. 1–3). The isolated spindles are highly extracted, free of membranes, and made up primarily of microtubules (Figs. 4–6). These isolated spindles can be stored and, for at least one species, reactivated to display microtubule assembly-disassembly characteristics similar to living spindles and to move chromosomes in a limited manner (Salmon and Segall, 1979, 1980).

Fig. 1. An early-anaphase spindle isolated from first-division eggs of the sea urchin Strongylocentrotus droebachiensis using an EGTA lysis buffer containing 1% Triton X-100 to solubilize the membrane components of the mitotic apparatus. The differential-interference contrast photomicrograph shows the various spindle parts and the separated chromosomes. The central spindle includes the regions between the chromosomes and nearest pole, called the half-spindle, and the region between the separating chromosomes, termed the interzone. The spindle poles abut the centrosome (CS)-centrosphere (CSPH) complex at the center of the asters. Astral fibers extend radially outward from the periphery of the centrosphere, which grows in diameter from metaphase through telophase and appears largely devoid of internal structure in electron micrographs, except for the centrosome complex surrounding the pair of centrioles (see Fig. 6). Bar = 10 μm.
5. MITOTIC SPINDLES ISOLATED FROM SEA URCHIN EGGS

Since Mazia and Dan first isolated the mitotic apparatus from dividing sea urchin eggs in 1952, several techniques have been developed for isolating stable spindles or mitotic apparatuses. A number of excellent detailed reviews of this work exist. Although one may not wish to employ the same isolation media used by these previous investigators, their reports are worth consulting because they contain a wealth of information on optimal procedures for culturing and preparing particular species of sea urchin eggs for spindle isolation.

This chapter will first briefly describe the major in vivo characteristics of the mitotic spindle. A brief historical review of isolation techniques will be followed by a description of the evolution of the EGTA lysis method that I use, emphasizing the influence on microtubule stability of buffer composition and intracellular physiological conditions. First-division sea urchin eggs are an excellent source for isolated spindles because large numbers of them can be cultured to develop synchronously. However, several important procedures must be mastered in order to remove the tough extracellular layers and obtain synchronous fertilization and development, and to determine the proper time for cell lysis, which I will consider next. We have found that culture procedures, cell lysis, solubility of the egg cortex, and spindle-microtubule stability in our EGTA lysis buffers are highly species specific; this report therefore will detail the procedures and results for the two sea urchin species with which I have had the most in-depth experience, Strongylocentrotus droebachiensis and Lytechinus variegatus.

II. In Vivo Spindle Characteristics

A. Definition of Spindle Parts

Some definitions and considerations of the in vivo mitotic spindle are in order. Detailed definitions can be found in McIntosh (1977). Mitotic apparatus refers to the chromosomes, the central spindle and astral fibers, the membrane components, and whatever other components are indigenous to the organelle in vivo. The term mitotic spindle refers specifically to the fibrous structures of the mitotic apparatus plus attached chromosomes and the mitotic centers. Parts of the mitotic

---

**Fig. 2.** Polarization micrographs of a living egg of Lytechinus variegatus at late metaphase (a) and mid-anaphase (b). The fertilization and hyaline layers have been removed, which reveals the radially positive birefringent retardation (BR) of the microvilli of the egg cortex. Polarization micrographs of spindles are taken with the interpolar axis of the spindle oriented at 45 degrees to the analyzer-polarizer directions and the compensator set for about 3.5 nm BR from the background light extinction. Astral fibers perpendicular to the spindle's interpolar axis appear dark in contrast because of the compensator. Note that the birefringent spindle sits within a clear zone from which the yolk particles have been excluded. The egg has been slightly flattened between the slide and coverslip. Bar = 20 µm.
spindle include the central spindle fibers, the kinetochores, the polar and interpolar fibers, the astral fibers, and the centrosome-centrosphere complex (see Fig. 1).

B. Spindle-Fiber Microtubules

In the living cell, forces for chromosome transport are generated along the spindle fibers, and most current theories of mitosis are based on proposed properties of the microtubules and associated complexes (Bajer, 1973; Inoué, 1976; Inoué and Sato, 1967; Inoué and Ritter, 1975; Margolis, 1978; Margolis et al., 1978; McIntosh, 1977; McIntosh et al., 1969; Nicklas, 1971, 1975, 1977).

Microtubules are the major structural elements of the spindle fibers (Fuge, 1978; McIntosh, 1977). Spindle-fiber microtubules tend to align in parallel arrays producing weak birefringent retardation (BR), about 1 nm. Dynamic changes in the number and distribution of spindle-fiber microtubules can be monitored using polarization microscopy methods (Inoué, 1964; Sato et al., 1975; Salmon and Ellis, 1976) in living cells and in isolated spindles (Figs. 2-4) (Hiramoto et al., 1981; Salmon and Segall, 1980).

The first-division spindles in sea urchin eggs are large. For example, the interpolar length of a metaphase spindle from *L. variegatus* is about 22 μm (Fig. 3); in *S. droebachiensis* eggs, it is about 25 μm depending on the temperature. Sea urchin chromosomes are small, and the kinetochore regions are not distinctly differentiated as in mammalian chromosomes. Although no detailed analyses have been done of microtubule distribution throughout mitosis in sea urchin embryos, some information is available. In most species of sea urchin there are about 38–44 chromosomes (Harvey, 1956; Hiramoto et al., 1981). Electron micrographs of isolated spindles have shown that the metaphase half-spindle contains 2500–3500 microtubules, depending on the conditions of fixation and the species of sea urchin (Cohen and Rehbun, 1970; Hiramoto et al., 1981; E. D. Salmon, unpublished observations). The number of kinetochore microtubules per chromosome is not well established, but about 15 are reported for *Arbacia* (Cohen and Rehbun, 1970), about 30 are estimated for the heart urchin *Clypeaster japonicus* (Hiramoto et al., 1981), and about 15 for *L. variegatus* (E. D. Salmon, unpublished observation). Microtubules attached to the kinetochores

---

**Fig. 3.** Changes in spindle-fiber BR and morphology induced by 2 μM Ca²⁺ in isolated metaphase spindles of *L. variegatus*. Isolated spindles in EGTA buffer (no glycerol) before treatment with 2 μM Ca²⁺ (a₁ and b₁) and 5 minutes after treatment (a₂ and b₂), viewed with polarization (a₁ and a₂) and phase-contrast microscopy (b₁ and b₂). The centrosomes (arrows) can be seen as phase-dense regions abutting the spindle poles. Note the loss of spindle-fiber BR (a₂) and the shortening of the chromosomal half-spindle fibers and astral fibers (b₂) that occurred after addition of 2 μM Ca²⁺. The width of the metaphase plate remains unchanged. Polarization micrographs were taken with the compensator set for 3.5 nm positive BR with respect to the spindle interpolar axis. (Modified from Salmon and Segall, 1980, with permission.) Bar = 10 μm; magnification, ×1200.
Fig. 4. A thin-section electron micrograph of the spindle pole-centrosome-aster complex of an early-metaphase spindle isolated from *L. variegatus*. Centrioles (CE) are contained within the centrosome complex (CS), which is a region where electron-dense material and ribosomelike particles are concentrated. At this stage a centrosphere structure is not apparent. Astral microtubules appear to end in the centrosome complex and extend radially away from it. The central-spindle microtubules do not appear to end in the centrosome complex, but terminate at a peripheral junction (J). (Modified from Salmon and Segall, 1980, with permission.) Bar = 1 μm.
5. MITOTIC SPINDLES ISOLATED FROM SEA URCWIN EGGS

appear to make up about 25-50% of the number of half-spindle microtubules. Most microtubules in a half-spindle are polar microtubules, which originate near the spindle pole and extend toward the chromosomes. Few extend from pole to pole; most end before reaching the chromosomes. Only a small percentage overlap the polar microtubules from the opposite pole (Hiramoto et al., 1981; E. D. Salmon, unpublished observation). In first-division eggs, the aster complexes grow during anaphase to occupy a greater volume than the central spindle (Fig. 1), but by the 32-cell stage, the asters are difficult to detect (Harris, 1962, 1975). The centrosome-centrosphere complex has not been well characterized.

C. Other Components

In the living cell the spindle region appears as a clear or achromatic zone (see Fig. 2) devoid of large cellular inclusions such as mitochondria or yolk particles. Microtubules contribute only 4% or less of the spindle volume. Exactly what components besides microtubules form a functional spindle fiber has not been determined (Forer, 1969, 1974; Forer and Goldman, 1972). Clearly, the mitotic

![Microtubules in an isolated spindle from *L. variegatus* seen in a thin-section electron micrograph at high magnification. Spindles were fixed by standard procedures with glutaraldehyde, postfixed with osmium tetroxide, and embedded in Epon-Araldite. The microtubule walls appear to be coated with a fine, fuzzy, filamentous network (filaments about 3-4 nm diameter) that can sometimes be seen to interconnect microtubules (white arrow). Thicker filaments (about 6 nm diameter) are also occasionally seen (black arrow). (From Salmon and Segall, 1980, with permission.) Bar = 0.1 μm; magnification, ×81,700.](image-url)
centers in the centrosomes or centrospheres are important, but their composition and structure are still poorly defined (see Figs. 4, 6). Actin and myosin filaments have been proposed as key functional elements of spindle fibers (Forer, 1974, 1976; Fujiwara and Pollard, 1978; Herman and Pollard, 1978, 1979; Sanger, 1977; Sanger and Sanger, 1976), as has a dynein-like intermicrotubule cross-bridging protein complex (Margolis, 1978; McIntosh, 1977; McIntosh et al., 1969; Pratt et al., 1980). In our isolated spindles there is a matrix of 3- to 4-nm, fuzzy, ill-defined filaments enmeshed with the spindle microtubules (Fig. 5).
This filamentous network may also be an important cytoskeletal component of
the spindle fibers, as may be the actin filaments (Figs. 6, 7).

Spindle microtubules are calcium labile (Fig. 3), and attention has been given
recently to the endogenous network of membrane vesicles, tubules, and cisternae
that has been observed in electron micrographs of fixed whole cells. The mem-
branes appear most noticeable near the spindle poles, filling the centrosphere in
first-division sea urchin spindles (Harris, 1975). Membrane tubules appear to
extend upward along the kinetochore fibers toward the chromosomes, and ra-
dially outward along the astral-fiber microtubules (Harris, 1975). This network
of smooth endoplasmic reticulum (SER) may function to sequester and release
calcium ions as does the sarcoplasmic reticulum of muscle (Harris, 1975, 1978a;
Hepler, 1977, 1979, 1980; Salmon and Segall, 1980). The calcium-binding
protein, calmodulin, has also been localized by immunofluorescence techniques
in the mitotic spindles of mammalian cultured cells with a distribution similar to
the SER (Andersen et al., 1978; Robbins and Jentzsch, 1969; Welsh et al.,
1979). It is a major protein of the sea urchin egg (Head et al., 1979). I decided
to eliminate the membrane components by using 1% Nonidet P-40 or 1% Triton
X-100 in the EGTA lysis buffers.

D. Microtubule Lability

Besides deciding which components not to preserve during spindle isolation,
the primary consideration of any isolation technique is to reversibly stabilize
the spindle microtubules. As in vivo spindle BR studies have established, spindle
microtubules are labile structures, particularly the non-kinetochore fiber micro-
tubules. They apparently exist in a constant state of flux with a cellular pool of
tubulin subunits (Cande et al., 1974; Inoué, 1964; Inoué and Sato, 1967; Inoué
et al., 1975; Rebhun et al., 1974a, 1975; Salmon, 1975; Salmon and Begg,
1980; Salmon and Segall, 1980). Spindle microtubules are reversibly depolymerized within several minutes by micromolar calcium, 4°C cooling, 400
atm pressure, or by 100 μM colcemid, colchicine, or other tubulin-binding
drugs, or metabolic inhibitors. In addition, the transport of chromosomes is
intimately coupled to the shortening and elongation of the spindle-fiber micro-
tubules (Inoué and Ritter, 1975; Nicklas, 1975, 1977; Salmon, 1975,
1976).

E. Egg Cortex

By first mitosis, the sea urchin egg cortex becomes highly differentiated.
Metaphase sea urchin eggs, stripped of the extracellular layers, look like fuzzy
tennis balls in scanning electron micrographs (Schroeder, 1978, 1979). Densely
packed microvilli cover the egg surface (see Fig. 2). The microvilli are structured
by a core of actin microfilaments that are anchored into the terminal web complex of the cortex, which is about 2 μm thick (Begg et al., 1978; see also Bryan and Kane, 1982). Cytoplasmic cleavage is produced by an actin-myosin-mediated cortical contractile process (Schroeder, 1975; Inoué and Kiehart, 1978). The mitotic spindle determines the plane of cleavage, but does not produce cleavage (Rapaport, 1975). Preservation of the actin filament structure of the egg cortex during lysis with EGTA lysis buffers is species specific, as will be discussed.

III. Approaches to in Vitro Spindle Models

There are three general approaches for providing direct access to the spindle machinery: (1) permeabilized models of mitotic cells; (2) cells lysed into tubulin-reassembly buffers; and (3) total isolation of the mitotic spindle into nontubulin buffers. A description of the permeabilized cell models is given by Cande (1982) in this volume. Since 1972, when Weisenberg showed how to reassemble microtubules in vitro, several methods have been developed that use exogenous brain MTP in microtubule-reassembly buffers to stabilize spindle microtubules after cell lysis. This work, as well as the earlier spindle-isolation techniques developed by Kane using hexylene glycol stabilization medium, has been clearly described and evaluated by McIntosh (1977). For growth of exogenous MTP on mitotic centers in lysed cell preparations, see Borisy and Gould (1977), Borisy (1982), and Kirschner (1979).

The properties of mitotic spindles isolated by the early methods developed by Mazia and co-workers and by Kane are reviewed in detail by Forer (1969). See also reviews by Turner and McIntosh (1977) and Zimmerman and others (1977). Zimmerman and co-workers (1977) also describe their isolation method, which uses DMSO-glycerol medium to stabilize microtubules principally in spindles from eggs of S. purpuratus. Sakai (1978a) has reviewed the biochemical properties of isolated spindles. He gives a clear appraisal of the microtubule composition and assembly characteristics and spindle ATPases, as well as a description of his method of isolating spindles in glycerol-isolation buffers. See also Pratt et al.

Fig. 7. Identification of actin filaments in isolated spindles of L. variegatus (a) and S. droebachiensis (b). Actin filaments were labeled in pellets of spindles using myosin-S, and the tannic acid fixation procedures of Begg et al. (1978). Actin filaments are seen in much higher concentration in the L. variegatus spindles. No bundles of actin filaments are seen in either case. Polarity of the actin filaments with respect to the chromosome-to-pole axis appears to be random. Many actin filaments are seen to extend along and to interconnect microtubules. (a) Bar = 1 μm; (b) bar = 0.1 μm. [These unpublished electron micrographs were provided by David Weiss (a) and Paul Browne (b), University of North Carolina, Chapel Hill, North Carolina.]

To obtain isolated mitotic apparatuses that include the endogenous, calcium-sequestering membrane components, see the method recently described by Silver et al. (1980).

IV. Evolution of ETGA Lysis Buffer

A. Historical Considerations

In 1972 Weisenberg reported that MTP purified from mammalian brain tissue could be reversibly assembled in vitro by using calcium-chelating reassembly buffers. Several laboratories then demonstrated that the microtubule structures of the mitotic spindle could be stabilized by appropriate concentrations of purified brain MTP (6S tubulin plus MAPs or HMW proteins) in the in vitro reassembly cell lysis buffers, or by the addition of high concentrations of glycerol, which had been shown to stabilize microtubule assembly in vitro (reviewed by McIntosh, 1977). Spindles stabilized in tubulin buffers or with glycerol maintained sensitivity to cooling for a short period of time following cell lysis, but in several cases the spindles were not rapidly depolymerized by dilution of the tubulin concentration. Sakai and co-workers were able to maintain a limited amount of chromosome movement following lysis of anaphase eggs into a complex microtubule-reassembly buffer that contained MES, EGTA, glycerol, Mg\(^{2+}\), GTP, ATP, tubulin, ascorbic acid, glutathione, cAMP, tubulin, and potassium fluoride (reviewed by Sakai, 1978a; see also Sakai, 1978b; Sakai et al., 1979).

Rebhun et al. (1974b) developed a procedure in which no exogenous tubulin, glycerol, or glycols were used to isolate sea urchin and surf clam mitotic apparatuses with relatively stable microtubules. Their lysis buffer contained, at pH 6.6–6.8: 0.1 M MES or PIPES, 1 mM EGTA, 0.1 M TAME (a proteolysis inhibitor), plus 0.2 M DTT and 0.1–1.0% Triton X-100 to lyse the cells. Cande et al. (1974) used similar procedures to obtain partially lysed tissue culture cells using 0.1–0.2% Triton X-100 in a reassembly buffer of 0.1 M PIPES, 1 mM EGTA, 1 mM GTP. Cande et al. (1974) noted that as the detergent concentration was increased, the rate of spindle dissolution decreased in tubulin-free reassembly buffer. One might expect high concentrations of detergent, which rapidly lyse the plasma and intracellular membranes, to promote rapid spindle dissolution as a result of the dilution of the cell’s tubulin concentration; however, the exact opposite occurred. Since the detergents Triton X-100 and Nonidet P-40 at 1% concentration do not alter the thermal lability of purified brain microtubules in vitro (Salmon and Segall, 1980), the reason for the stability of the spindle microtubules in Rebhun’s and Cande’s lysis buffers was not clear.
B. EGTA Lysis Buffer

In view of the wide variety of techniques published, I decided to look for minimal buffer conditions that would permit isolation of mitotic spindles with relatively stable microtubules. In 1977 Robert Jenkins and I found that mitotic spindles from first-mitotic sea urchin eggs of *S. droebachiensis* or *L. variegatus* could be isolated with a relatively stable normal distribution of spindle BR and organization of microtubules by rapidly lysing the eggs (plus fertilization membranes) in a simple calcium-chelating (EGTA), low-ionic-strength, Triton X-100 detergent buffer. Critical requirements for microtubule preservation during cell lysis were strong calcium chelation, low ionic strength, rapid membrane solubilization, and pH below 7.0. Adding micromolar concentrations of calcium ions or raising the KCl concentration above 0.3 M or the pH above 9.0 rapidly dissolved the spindle BR. Table I summarizes the stability of spindle BR as a function of various lysis buffer conditions we tried. Our standard EGTA lysis buffer now contains 5 mM EGTA, 0.5 mM MgCl₂, 10-50 mM PIPES (pH 6.8-7.0), plus 0.5-1.0% Triton X-100 or Nonidet P-40. As described below, glycerol can be added to the EGTA lysis buffer to stabilize the spindle microtubules reversibly during cell lysis and spindle storage (Sakai and Kuriyama, 1974).

C. Physiological Considerations

In the living cell, steady-state free-calcium-ion concentration is probably below $10^{-7}M$. Elevation of the free-calcium-ion concentration above 1 $\mu M$ rapidly depolymerizes sea urchin spindle microtubules (Inoué and Kiehart, 1978; Kiehart, 1979, 1982; Salmon and Segall, 1980). The pH of the sea urchin may be between 7.0 and 7.6—an exact value has not yet been established (Johnson *et al.*, 1976; Shen and Steinhart, 1978; Steinhart *et al.*, 1978). Very little spindle BR was preserved with our EGTA lysis buffer at pH 7.4. Isolation at pH 6.6 preserved BR well, but with considerable cytoplasmic contamination and over-stabilization of the spindle microtubules. We compromised on pH 6.8-7.0. Extraordinarily clean isolated spindles could be obtained in magnesium-free isolation buffers (10-30 mM EDTA added to the EGTA lysis buffer), but the microtubules were extremely labile and the spindles difficult to purify. We use 0.5 mM MgCl₂ in our isolation buffer to promote microtubule stability. Magnesium ions, however, also promote nonspecific cytoplasmic contamination and stability of the egg cortex during cell lysis, so that higher concentrations of Mg²⁺ are not useful. The osmolarity and ionic strength of the sea urchin cytoplasm is high. The ionic composition of the egg, although not well defined, appears to include more than 0.3 M glycine, about 0.25 M K⁺, about 30-50 mM Cl⁻, and other anions, perhaps 0.2 M glutamate (Baker and Whitaker, 1979; Kavanau, 1953;
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Buffer composition</th>
<th>Isolation temp. (°C)</th>
<th>Initial BR (nm)</th>
<th>Half-life BR (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>1 mM EGTA, 1 mM MgCl₂, 100 mM MES (pH 6.6)</td>
<td>15</td>
<td>5.6</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1 mM EGTA, 100 mM PIPES (pH 6.8)</td>
<td>15</td>
<td>5.0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1 mM EGTA, 100 mM sodium cacodylate (pH 7.1)</td>
<td>15</td>
<td>3.5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1 mM EGTA, 1 mM MgCl₂, 10 mM Tris (pH 8.0)</td>
<td>15</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>1 mM EGTA, 10 mM MgCl₂, 0.1 M KCl, 10 mM NaH₂PO₄ (pH 7.2)</td>
<td>15</td>
<td>2.8</td>
<td>2</td>
</tr>
<tr>
<td>% Detergent</td>
<td>1 mM EGTA, 1 mM MgCl₂, 100 mM PIPES (pH 6.8), 0.025% Triton X-100</td>
<td>15</td>
<td>3.5</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>As above with 0.075% Triton X-100</td>
<td>15</td>
<td>3.5</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>As above with 0.25% Triton X-100</td>
<td>15</td>
<td>5.6</td>
<td>2</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>1 mM MgCl₂, 50 mM PIPES (pH 6.8)</td>
<td>15</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>5 mM EGTA, 10 mM PIPES (pH 6.8)</td>
<td>15</td>
<td>5.6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10 mM EGTA (pH 6.8)</td>
<td>15</td>
<td>5.6</td>
<td>1</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>5 mM EGTA, 10 mM EDTA, 10 mM PIPES (pH 6.8)</td>
<td>15</td>
<td>5.0</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>5 mM EGTA, 0.5 mM MgCl₂, 10 mM PIPES (pH 6.8)</td>
<td>15</td>
<td>5.6</td>
<td>2</td>
</tr>
<tr>
<td>Temperature</td>
<td>1 mM EGTA, 1 mM MgCl₂, 100 mM MES (pH 6.6)</td>
<td>22</td>
<td>6.3</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>As above at</td>
<td>15</td>
<td>5.6</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>As above at</td>
<td>8</td>
<td>3.5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>As above at</td>
<td>0</td>
<td>0.7</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*Spindles are from eggs grown at 8°C.

*Note: Eggs were cleaned of their jelly coat, fertilized, grown to first-division metaphase, then rapidly diluted 100-fold into lysis buffer. Preserved spindles and lysed cytoplasm were retained within the fertilization membrane, which was easily held between slide and coverslip for observation in the polarization microscope. BR measurements are averages of 10 or more spindles (S.D. = ±15%).

*All buffers contained 0.25% Triton X-100 unless otherwise stated.

*EGTA buffer referred to in text.
Rothchild and Barnes, 1953; Schatten and Mazia, 1976). The osmolarity of seawater is about 1.1, depending on local conditions. As seen in Table I, spindles were poorly preserved when lysed in media with high ionic strength. Therefore, before cell lysis, we wash cells free of growth medium with a nonionic isosmotic medium (we use 1 M glycerol, 5 mM Tris, pH 8.0). If this is not satisfactory, try 1 M dextrose (Sakai et al., 1977), or an isonic medium of 19 parts NaCl to 1 part KCl to remove divalent cations (Table II). During lysis, eggs should be rapidly diluted 100-fold (v/v) into lysis buffer to make certain that the components of the egg cytoplasm do not raise the calcium concentration or ionic strength, or lower the pH.

D. Species Specificity

I have found that the properties of the EGTA-isolated spindle preparations vary with different species. For instance, highly purified spindle preparations suitable for biochemical analysis can be obtained from S. droebachiensis, but the spindle microtubules from this urchin are unusually stable following lysis and washing. In contrast, spindles from L. variegatus are highly labile to calcium ions (Fig. 3) and can be treated to display lifelike thermal lability and a limited shortening of spindle fibers and chromosome movement. L. variegatus spindles are suitable for physiological or antibody-labeling studies of single spindles, but the spindle preparation also contains well-preserved egg cortices, containing actin filaments in high concentration. Thus for L. variegatus the EGTA lysis buffer yields a relatively stable whole-cell “cytoskeleton.” In a biochemical analysis of the spindle proteins in a preparation of L. variegatus spindles, the spindle proteins are only a minor component. Why the spindle microtubules and egg cortices of different species have such striking differences in stability is not known, but the phenomenon is a major consideration in the choice of a biological source for isolated spindles. One can expect many other features of the isolation procedure and resulting spindles to be peculiar to individual species.

E. Other Cell Types

Over the past several years I have briefly examined several other cell types for the ability of EGTA lysis buffers to produce highly extracted spindles with relatively stable spindle-fiber BR. Successful results were obtained for the marine eggs of Echinarachnius parma (sand dollar), Asterias forbesi (starfish), Arbacia punctulata (sea urchin), and Spisula (surf clam) (see also Murphy, 1980; Rebhun et al., 1974b; Sakai et al., 1977). Nonmarine sources of isolated spindles have included PtK, tissue culture cells, Haemanthus endosperm cells, and the ciliate Spirostomum (intranuclear spindles of micronuclei) (Salmon and Jenkins, 1977).
V. Procedures for Isolating Spindles

A. General Considerations

To isolate first-mitotic spindles from developing sea urchin eggs the following steps must be accomplished (see Fig. 8).

1. Obtain ripe sea urchins.
2. Shed gametes.
3. Remove the egg jelly coat.
4. Fertilize all the eggs synchronously without inducing polyspermy.
5. Remove the tough extracellular coats—the fertilization membrane and hyaline layer.
6. Culture developing eggs to first division synchronously (generally in calcium-free seawater at constant, species-specific temperature).
7. Identify correctly the mitotic stage where isolation procedures are to be executed.

![Diagram showing the procedure of isolating spindles](image)

**Fig. 8.** Schematic drawing of the procedures for making detergent-extracted cytoskeletons and isolated spindles from the embryos of *L. variegatus*. Procedures for *S. droebachiensis* embryos are basically similar, but have several important differences in detail, as described in the text.
8. Wash the eggs into an iso-osmotic medium free of divalent cations (and low ionic strength if the eggs are not adversely affected).
9. Lyse the eggs into isolation buffer, and collect spindles by low-speed centrifugation.
10. Wash spindles in lysis buffer, then store spindles in an EGTA–glycerol buffer.

Note that sea urchins ripen on a seasonal basis in their natural habitat, hence it is best to work at a marine laboratory. Although methods for maintaining ripe sea urchins or ripening them in artificial seawater away from the marine laboratory are in general use, there is not, to my knowledge, any detailed published summary of the procedures used. Commercial sources of sea urchins include the following:

1. Marine Biological Laboratories, Woods Hole, Massachusetts 02543
   Arctica punctulata (June–Aug.), S. droebachiensis (Feb.–April)
2. Gulf Specimen Company, Box 237, Panacea, Florida 32346
   L. variegatus (May–Oct.), Arctica punctulata (May–Oct.)
3. Marine Specimens Unlimited, R.R. 2, Box 879, Summerland Key, Florida 33042
   L. variegatus (March–Sept.)
4. Pacific Bio-Marine Laboratories, Inc., P.O. Box 536, Venice, California 90291
   S. purpuratus (Dec.–May), L. pictus (May–Sept.)

I have indicated within the parentheses my experience as to the seasonal availability of ripe sea urchins from the above suppliers. Other species may also be available.

Special attention should be given to maintaining nearly the same salinity in the artificial seawater as the native salinity of the organisms. This is usually nearer to 31‰ rather than the 34–35‰ normally given for the open ocean. See Cavanaugh (1956) and Table II for formulas for artificial seawaters.

Procedures for obtaining gametes from ripe sea urchins; for fertilization; for removal of the egg jelly coat, fertilization membrane, and hyaline layer; and for development all are species dependent, yet well established. The best general manuals available are those of Costello and Henley (1971) and Harvey (1956). Harvey also gives an in-depth description of the biology of the sea urchin. Most of the current methods for culturing eggs for spindle isolation were devised by Mazia and co-workers and by Kane and co-workers (see refs. given previously).

A major factor to be considered in isolating spindles from sea urchin eggs is that the jelly coat and the tough extracellular layers that form around the egg on fertilization must be removed without damaging synchronous development (for review see Epel, 1977). Ripe eggs are covered by a thick jelly coat that hydrates
TABLE II
Composition of Buffers Used in Isolation of Mitotic Spindles from L. variegatus and S. droebachiensis

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artificial seawater*</td>
<td>423 mM NaCl, 9 mM KCl, 23 mM MgCl₂·6H₂O, 25 mM MgSO₄·7H₂O, 2 mM NaHCO₃, 9 mM CaCl₂·2H₂O, 5 mM Tris (pH 8.0-8.3)</td>
</tr>
<tr>
<td>Calcium-free artificial seawater*</td>
<td>436 mM NaCl, 9 mM KCl, 34 mM MgCl₂·6H₂O, 16 mM MgSO₄·7H₂O, 1 mM EGTA, 5 mM Tris (pH 8.0-8.3)</td>
</tr>
<tr>
<td>Glycerol buffer</td>
<td>1 M glycerol, 5 mM Tris (pH 8.0-8.3)</td>
</tr>
<tr>
<td>19:1 Isotonic buffer</td>
<td>560 mM NaCl, 30 mM KCl, 5 mM Tris (pH 8.0-8.3)</td>
</tr>
<tr>
<td>EGTA Buffer</td>
<td>0.2-5.0 mM EGTA, 0.5 mM MgCl₂, 10 mM PIPES (pH 6.8-7.0)</td>
</tr>
<tr>
<td>EGTA Lysis buffer</td>
<td>5 mM EGTA, 0.5 mM MgCl₂, 10-50 mM PIPES (pH 6.8-7.0); 1% Triton X-100 or 1% Nonidet P-40, 20% (v/v) glycerol (optional)</td>
</tr>
<tr>
<td>EGTA-Glycerol storage buffer</td>
<td>5 mM EGTA, 0.5 mM MgCl₂, 10 mM PIPES (pH 6.8); 50% (v/v) glycerol, 1 mM DTT</td>
</tr>
<tr>
<td>Reassembly buffer</td>
<td>1 mM EGTA, 0.5 mM MgCl₂, 0.5 mM GTP, 100 mM PIPES (pH 6.8)</td>
</tr>
</tbody>
</table>

*Modified Moore calcium-free artificial seawater (Cavanaugh, 1956).

and swells on shedding into seawater. Jelly layers 100 µm thick are not uncommon. After sperm-egg membrane fusion, the cortical granules fuse with the egg plasma membrane and release their contents within the perivitelline space. This raises the vitelline membrane up off the egg surface to become the fertilization membrane. In addition, enzymes are released that cross-link and harden the fertilization membrane within 1-2 minutes. The egg jelly may be removed by using calcium-free seawater, by lowering pH to 5.25, or by mechanical shearing; the hardening of the fertilization membrane may be prevented by using either 1 M glycerol or 1 M urea, or by pretreatment of the unfertilized eggs with pronase, trypsin, or DTT (Begg and Rebhun, 1979; Epel, 1970; Kane, 1962, 1965, 1967; Mazia et al., 1972; Pratt et al., 1980; Salmon and Segall, 1980; Stephens, 1972b, 1973; Zimmerman et al., 1977). Following fertilization, the egg secretes an extracellular layer called hyaline. In some urchins (e.g., Arbacia, Lytechinus) it is highly insoluble; however, it requires calcium to harden. Consequently, we grow fertilized embryos in calcium-free seawater to prevent formation of the hyaline layer. Some urchins do not secrete excessive amounts of hyaline (e.g., S. droebachiensis and S. purpuratus), so for these sea urchins regular artificial seawater may be used.

The development of ripe sea urchin eggs is generally uniform and synchronous under a constant set of conditions: temperature, handling of the eggs, composition of culture medium, and so on. Development rates are very sensitive to temperature (Q₁₀ ~ 2.5-3.0). Costello and Henley (1971) or Harvey (1956) is a good source of information for timetables. Fry (1936) gives an excellent pictorial
analysis of developmental timetables for *Arbacia* at several temperatures. Stephens (1972a) also provides a similar analysis for *S. droebachiensis*. We have found that the developmental timetables for *L. variegatus* are similar to those reported by Fry for *Arbacia*. However, because procedures and culture media differ, it is best to generate a custom timetable for development of specific sea urchins under your laboratory conditions. Controlling temperature for all media used is of the utmost importance if timetables are to be useful.

Temperature is also an important parameter because it affects the extent of spindle microtubule assembly. The optimal temperature for development and spindle assembly is highly species specific. As seen in Fig. 9, *S. droebachiensis* and *L. variegatus* must be grown at distinctly different temperatures. Based on spindle BR measurements and development, the optimal temperature for *S. droebachiensis* is 7–9°C (Stephens, 1972b, 1973), whereas the optimal temperature for *L. variegatus* is 23–25°C. *S. droebachiensis* lives and spawns in the cold northern Atlantic waters, whereas *L. variegatus* is tropical, found on the Atlantic coast usually south of Virginia.

To isolate spindles it is also important to identify accurately the mitotic stage. Polarization microscopy is the most useful method (see Fig. 2) if the egg is

![Graph](image)

**Fig. 9.** The effect of temperature on metaphase spindle BR in developing eggs of *S. droebachiensis* (o, SD) and *L. variegatus* (●, LV). *L. variegatus* eggs were grown initially at 22.5°C and *S. droebachiensis* eggs were grown at 8°C. After the eggs reached prometaphase they were transferred to the experimental temperature and the spindle BR was measured (Stephens, 1973; Salmon, 1975; Salmon and Ellis, 1976). Single arrow (12–14°C) indicates the upper temperature limit of development for *S. droebachiensis*, and double arrow (33–34°C) indicates the inhibitory temperature for *L. variegatus*. The data for *S. droebachiensis* were derived from Stephens (1973); data for *L. variegatus* are from E. D. Salmon (unpublished). Data points represent average values. BR range ±15% of average values.
relatively clear like *L. variegatus* or *S. droebachiensis*. Identifying mitotic stage without a polarization microscope is a difficult problem in pattern recognition. Because the spindle actively excludes large cellular inclusions, the spindle can be viewed with a bright-field, phase-contrast, or differential-interference contrast microscope as a clear zone that changes in morphology along with the spindle as mitosis proceeds (Fig. 2). Harris (1978b) gives a series of light micrographs of fixed preparations of *S. purpuratus* that should be instructive. Another method of identifying the mitotic stage is to time the development of the embryos after fertilization; however, this is rarely sufficiently accurate because variations in development rate occur between different batches of eggs.

The procedure for handling eggs and for isolating spindles must be tailored for each species of sea urchin. Described here first are the procedures I use for isolating spindles from *L. variegatus*. The procedures for isolating spindles from *S. droebachiensis*, described second, differ principally in the methods used to remove the egg jelly, fertilization membrane, and the hyaline layer. The current spindle-isolation procedures were derived from information and results reported by Rebhun *et al.* (1974b), Kane (1962, 1965, 1967), Stephens (1972b), and Sakai and co-workers (Sakai and Kuriyama, 1974; Sakai *et al.*, 1975, 1977).

### B. Methods for *L. variegatus*

#### 1. Collecting Gametes

Extensive spawning is induced by injecting 1–3 ml of 0.56 M KCl into the sea urchin’s body cavity. Alternately, Fuseler (1973) has described a technique for obtaining small numbers of gametes repetitively from sea urchins over a long duration. If the urchin is a male, the sperm is collected “dry” (i.e., by not allowing the urchin to shed into seawater). The concentrated sperm can be pipetted into a test tube on ice; or the whole urchin can be inverted over a watch glass sitting on ice; or the testes can be excised and stored at 4°C and the sperm will be secreted slowly around the outside of the testis. Dry sperm, free of coelomic fluid contamination, will remain viable when diluted into seawater for 1 or 2 days. Female sea urchins are inverted over a 100-ml beaker filled to the brim with seawater at physiological temperature (20–30°C for *L. variegatus*); usually room temperature is satisfactory. The sea urchin is allowed to spawn for 10–15 minutes. The last eggs spawned tend to be immature, and these should be avoided.

#### 2. Ripeness

At this time, some decision about the quality of the eggs should be made. Unripe urchins produce few eggs and may spawn many unripe oocytes. In
immature oocytes, the large germinal vesicle is very apparent. The KCl treatment will also cause overripe urchins to spawn eggs that are in a state of deterioration, readily identified by their brownish-red appearance and by the presence of many egg fragments. Ripe sea urchin eggs (about 125 μm diameter for *L. variegatus*) are arrested at the end of meiosis II, have released both polar bodies, and the female pronucleus sits up against the egg cortex at the cell surface. A ripe urchin should yield 10–30 ml of packed eggs containing their jelly coats.

3. **Egg Jelly**

Removal of the egg jelly will reduce the volume of the egg pellet more than 10-fold. The jelly layer is removed from *L. variegatus* eggs, immediately after shedding, by decanting the eggs in 100 ml of seawater through a 150-μm-pore Nitex screen (Tetko, Inc., Elmsford, New York). A dense sample of eggs that no longer have their jelly coats will be seen on a slide in the dissection scope to touch each other. Otherwise, they will be spaced by the thickness of the jelly layers. If the egg jelly is not released by screening in normal seawater, substitution of calcium-free seawater (Table II) during the screening should solve the problem. Complete removal of the jelly coat is critical for preservation of spindle BR during isolation procedures. After screening, the eggs are washed twice in 100 ml of artificial seawater by letting them settle and decanting the seawater. Eggs will fertilize and develop normally if used within 8 hours of the washing process.

4. **Fertilization and Removal of the Fertilization Membranes**

Check fertilizability and have a control for timing mitotic events by starting a small sample 10 minutes ahead of the large batch. The procedures described here must be done rapidly, on schedule, without interruption. We frequently use four 15-ml conical centrifuge tubes and a hand centrifuge to pellet the eggs. An aspirator with a Pasteur pipet tip is used to remove the supernatants quickly.

A comment is necessary about the concentration of sperm used for fertilization. If the concentration is too low, not all the eggs will be rapidly fertilized; too high, more than one sperm will enter an egg, yielding polyspermy. A polyspermic egg will generally have multipolar spindles and may not develop at the same rate as monospermic eggs. The procedures given here include a sperm dilution we have found to be optimal, but the dilution may have to be altered for different laboratory conditions. We dilute one drop of dry sperm into 12 ml of seawater near 23–25°C (the best developmental temperature for *L. variegatus*). One milliliter of diluted sperm is used to fertilize about 1 ml of packed eggs suspended in 60 ml of seawater. After 30 seconds, the eggs are gently pelleted in the hand centrifuge (this takes about 20 seconds), and the supernatant is quickly aspirated (20–30 seconds) and replaced by 1 M glycerol–5 mM Tris, pH 8.3 (glycerol
buffer) (20 seconds) to soften and remove the elevating fertilization membrane. The fertilized eggs are gently pelleted in the hand centrifuge (speed is no longer necessary), resuspended in glycerol buffer again, and after 1 minute in glycerol buffer the eggs are gently pelleted and resuspended in calcium-free artificial seawater (Table II).

5. Embryo Development

The fertilized eggs are grown in calcium-free artificial seawater in large flat-bottom finger bowls (~ 20 cm diameter) as monolayers. About 1 cm of calcium-free seawater should cover the eggs. (Calcium-free seawater is used to prevent formation of the extracellular hyaline layer.)

The development stage is established by observing an aliquot of eggs with a low-power polarization microscope or bright-field microscope (defocus condenser from Koehler illumination for bright field to increase the contrast of the yolk-free spindle region). At 23–25°C, L. variegatus eggs should reach metaphase 50–60 minutes after fertilization. Timing, as mentioned above, is extremely dependent on temperature.

6. Lysis

About 5 minutes before the desired stage for spindle isolation, the embryos are collected into 15-ml centrifuge tubes. Excess medium is aspirated off the monolayer of eggs in the finger bowls and the eggs are collected into four 15-ml conical centrifuge tubes by several centrifugations (gently, to avoid premature egg lysis). One minute before lysis, the eggs are suspended in isotonic glycerol buffer to remove the seawater salts. Then they are pelleted and about 0.25 ml of pelleted eggs is resuspended rapidly into 12–15 ml of lysis buffer. The EGTA lysis buffer for L. variegatus is given in Fig. 8 and Table II. Spindles are freed from the cortices, with difficulty, by vigorous pipetting. After 15 minutes in the lysis buffer, the isolated cytoskeletons (spindle plus cortex), spindles, and cortices are pelleted in a clinical centrifuge at 500 g for 10 minutes; resuspended in a glycerol storage buffer (Table II); then stored at 4°C. We have kept spindles for as long as 5–6 weeks without significant deterioration, but the maximum possible length of storage has not been determined. Spindles can be satisfactorily shipped between labs in the glycerol storage buffer on ice or, for biochemical analysis, on dry ice.

When L. variegatus spindles are isolated in EGTA lysis buffer without glycerol, initial spindle BR is nearly identical to the BR of spindles in the living cell before lysis; it decays slowly in EGTA buffer, however, reaching half its initial value in 30–45 minutes. For L. variegatus eggs, consequently, I add glycerol to the isolation buffer to stabilize the microtubules during the lysis,
washing, and storage procedures. When ready to use the spindles for experiments, I wash out the glycerol with EGTA buffer, thus restoring the normal lability of isolated L. variegatus spindles.

C. Methods for S. droebachiensis

Whereas L. variegatus embryos are grown at 23–25°C, S. droebachiensis embryos develop best at 7–9°C. All solutions should be kept at this low temperature except for the lysis buffer.

1. Obtaining Gametes

Procedures are basically the same as for L. variegatus, except that the females should be shed into 100-ml beakers containing seawater on ice. Allow the urchins to shed for 20–30 minutes. The ripe egg is about 175 μm in diameter. One ripe female should yield about 30–40 ml of packed eggs with jelly coats. Eggs of S. droebachiensis are much more fragile than those of L. variegatus.

2. Fertilization, Removal of Egg Jelly and Fertilization Membrane

Twenty to thirty milliliters of eggs (including jelly coats) are suspended into an equal volume of seawater at 8°C in a 100-ml glass beaker; then they are fertilized with about 10 drops of diluted semen (3 drops of dry sperm in 12 ml of seawater). Ninety seconds after fertilization a control sample is taken to check the percentage of fertilization. Two minutes after the addition of sperm, a pH electrode is inserted into the egg suspension, which is stirred with a glass rod. At 2.5 minutes the pH of the seawater is quickly reduced to 5.25 (not less!) by pipetting in 0.1 N HCl while stirring constantly (this should be done within 30 seconds). This removes the egg jelly. The eggs are then pelleted gently by hand centrifugation (about 30 seconds) and washed twice in glycerol buffer (speed is no longer important) to remove the fertilization membranes. Finally, the eggs are resuspended in seawater near 8°C and plated out into large finger bowls as described earlier for L. variegatus eggs.

3. Lysis

About 5 minutes before the time of isolation, eggs are collected into four 15-ml centrifuge tubes and washed twice in an isotonic solution (19 parts NaCl to 1 part KCl) to remove divalent cations and to weaken any hyaline layer (this is not usually troublesome with S. droebachiensis eggs). The pelleted eggs are lysed in an EGTA lysis buffer without glycerol at 19–22°C (note the warmer temperature now) and shaken vigorously to release the mitotic spindles and to
disperse the cortices. Unlike *L. variegatus* eggs, the cortex of *S. droebachiensis* rapidly solubilizes in this low-ionic-strength EGTA lysis buffer so that the spindle structures are the dominant elements in the lysate. After 30 minutes of lysis, whole eggs and fertilization membranes can be removed by filtering the preparation with a 50-µm-pore Nitex screen. Spindles are then pelleted at room temperature in a clinical centrifuge for 10 minutes at 500 g. The pellet is washed once in 1.5 ml of EGTA lysis buffer. For biochemical analysis, washed pellets are directly resuspended in the desired analysis medium. Since the BR of *S. droebachiensis* spindles is very stable in EGTA lysis buffer alone, no glycerol is necessary to stabilize spindles during the lysis and washing process. However, to store the spindles, they are kept in the EGTA-glycerol storage buffer developed for *L. variegatus* spindles. Stored spindles remain birefringent for several months when kept at −20°C. *S. droebachiensis* can be isolated and washed at 8°C instead of 19–22°C. Such preparations appear similar in protein composition on SDS-PAGE gels, but yield less microtubule polymer (Pratt et al., 1980).

VI. Characteristics of Spindles Isolated in EGTA Lysis Buffers

The characteristics of both the *L. variegatus* and *S. droebachiensis* isolated spindles are described in detail elsewhere (Salmon and Segall, 1980; Pratt et al., 1980), so will be presented only briefly here. As mentioned before, many characteristics of the isolated spindles are species dependent.

A. Structural Characteristics

The spindles from both species, when isolated in EGTA lysis buffer, are highly extracted, membrane free, and made up primarily of microtubules (Figs. 4, 5, 6, 7). With lower concentrations of detergent in the EGTA lysis buffer (0.25–0.50%), membrane sheets and vesicles are present in the isolated preparations. Actin filaments have been identified by myosin-S1 labeling in both species (Fig. 7), but whether this actin is endogenous to the spindle or filters into the spindle during lysis is an unresolved question. Unknown ribosome-like particles and clusters of particles are distributed along and among the microtubules. The density of these particles appears highest in the centrosome complex, particularly for spindles isolated from *S. droebachiensis* eggs (Figs. 4, 5). The particles also appear to be associated with a fuzzy network of 3- to 4-nm filaments that do not decorate with myosin-S1, but form a microtrabecular matrix in which the microtubules are embedded.

The pattern and distribution of BR is typical of mitotic spindles in living cells. In *S. droebachiensis* isolated spindles, swelling of the centrospheres was noticeable during lysis in the low-ionic-strength EGTA buffer, but the amount has not
been determined. Immediately after lysis without glycerol, the magnitude of BR for *L. variegatus*, measured in the central half-spindle region, is about 2.5–3.0 nm for metaphase spindles at 23°C. The measured BR in living cells is about 25% lower. Based on form birefringence calculations, the microtubule BR of the spindles in living cells is expected to be 0.71–0.83 times the value in EGTA lysis buffer because of the difference in the refractive index of the surrounding medium (1.352–1.364 for the living cytosol versus 1.333 for EGTA buffer) (Hiramoto *et al*., 1981; Sato *et al*., 1975). The BR of the *S. droebachiensis* isolated spindles is somewhat higher than this ratio would predict from the BR measured in living cells (5–6 nm versus about 3 nm). This increased BR over the expected value may reflect microtubule polymerization during the lysis procedure, or—more likely—the concentration of ribosome-like particles and amorphous material that appears to be aligned along the microtubules and to coat them. The density of this material, which is higher than in *L. variegatus* spindles, appears species specific. It does not depend on the presence or absence of glycerol, but has been seen to vary in density with the concentration of 

\[ \text{Mg}^{2+} \] in the EGTA lysis buffer (E. D. Salmon, unpublished observation).

During isolation the spindles frequently are broken in two distinct locations: at the interzone of anaphase spindles and at the junction between the central spindle poles and the aster complex (see Figs. 3, 4, 11). The mechanical weakness of the isolated spindle in the interzone is understandable because there are significantly fewer microtubules in the interzone in comparison with the half-spindles, as indicated by the distribution of BR in Fig. 2. The aster complex appears to be broken off the ends of the half-spindle easily because the half-spindle microtubules are not anchored in the centrosome as is usually described for mammalian spindles (see McIntosh, 1977, 1982). As seen in Fig. 4, the half-spindle microtubules terminate at a noticeable boundary zone at the periphery of the aster complex. This structural discontinuity in the spindles merits further attention because it suggests that detergent-solubilized components may be the mitotic centers for organizing the central-spindle microtubules.

Another interesting feature of the EGTA-isolated spindles is the spindle "remnant." As discussed below, calcium concentrations above 2 \( \mu M \) cause the microtubules of the isolated spindles to be completely depolymerized. However, after the BR of the spindle fibers has disappeared, a remnant of the spindle can still be seen with phase-contrast microscopy (Figs. 3, 11). We have not yet completely characterized this remnant, but it appears to be composed largely of the 3- to 4-nm fine filaments and the ribosome-like particles.

### B. Biochemical Characteristics

Because the cortices of *L. variegatus* eggs are well preserved when the spindles are isolated, we have not pursued any biochemical analysis of the preparations. In contrast, only a low percentage of cortex-actin filament complexes
Fig. 10. 5% Tris-glycine-buffered SDS–polyacrylamide gels of *S. droebachiensis* isolated mitotic spindles (MS), whole eggs (WE), and blastula cilia (C). α- and β-Tubulin subunits are the dominant protein components of the isolated spindle, whereas they are barely detectable in the whole egg. No 22S or 2.5S cytoplasmic yolk protein and little actin (43K) from the cortex contaminate the spindle preparation. An approximate molecular weight scale is at the left ($\times 10^3$ daltons). (From Pratt *et al.*, 1980, with permission.)

can be seen in electron micrographs of pelleted spindle preparations from *S. droebachiensis*. *S. droebachiensis* has proved to be an ideal source of native spindle proteins. At 20°C, lysis of 60 mg (0.6 ml) of first-division eggs yielded 0.3 mg of spindle protein, or 0.5% of the total egg protein. As seen from
SDS-PAGE analysis (Fig. 10), α- and β-tubulin make up at least 20% of the total spindle protein. Little 22S yolk protein contaminated this preparation. An unidentified 55,000-dalton protein, not tubulin, is a significant component (about 5%); it may be an element of the 3- to 4-nm filaments that enmesh the microtubules. Actin is usually only 2–8% of the total protein, indicating little cortical contamination of the spindle preparation. Two HMW bands (about 300,000–400,000 daltons) have been identified to be similar to cytoplasmic egg dynein and a dynein-like Mg-ATPase activity has been demonstrated (Pratt et al., 1980). No evidence was found for myosin ATPase, ouabain-sensitive Na⁺/K⁺ ATPase, or oligomycin-sensitive mitochondrial ATPase or the mitotic Ca²⁺-ATPase. Since the Ca²⁺-ATPase is believed to be bound to membranes, one would not expect to find it in detergent-extracted spindles (Mazia et al., 1972; Nagle, 1979; Petzelt, 1972, 1979; Petzelt and Auel, 1978; Petzelt and von Ledebur-Villiger, 1973).

C. Physiological Characteristics

The isolated spindles of *S. droebachiensis* are unusually stable. The spindle-fiber BR remains stable for hours at room temperature in EGTA buffer without any exogenous tubulin or glycerol, and it is not significantly altered by cooling to 4°C or pressurization to 8000 psi. However, 0.4 M KCl rapidly abolishes the spindle BR, which may indicate that low ionic strength has a profound stabilizing effect on the *S. droebachiensis* spindles.

The isolated spindles from *L. variegatus* are labile and have been useful for studying variables that control microtubule assembly and spindle-fiber shortening. (Spindles from the sea urchin *Arbacia* have properties similar to those of *L. variegatus.*) For physiological studies on isolated spindles, I sandwich a drop of the isolated spindle preparation in glycerol storage buffer between an ethanol-cleaned slide and coverslip (previously rinsed in deionized water). The coverslip is supported by ridges of silicone grease (Dow Corning Corp., Midland, Michigan). After 10–15 minutes, many spindles will have settled and become weakly stuck to the slide. Fragments of #1 filter paper are then used to draw buffers through the slide-coverslip preparation. Experiments are done on a sensitive polarization microscope that has been modified so that phase-contrast or differential-interference contrast optics can be switched in without moving the specimen (Salmon and Segall, 1980). Most routine recording of the changes in spindle morphology is done using time-lapse video-recording techniques. When stored spindles are transferred out of EGTA-glycerol storage buffer into an EGTA or microtubule-reassembly buffer (Table II), the initial BR, rate of BR decay (half-life ~ 30 minutes), and calcium lability are similar to freshly isolated spindles in the absence of glycerol.

A major feature of the EGTA-isolated spindle is its calcium lability. Calcium
in low micromolar concentrations has two distinct effects on the isolated spindles (Fig. 3). First, spindle BR decays as microtubules depolymerize rapidly, the rate increasing with calcium concentration above 0.2–0.5 μM, as assayed using Ca–EGTA buffers. Microtubules depolymerize almost completely in less than 6 minutes at 2 μM Ca^{2+}, and within several seconds at 10 μM Ca^{2+}. Second, concurrent with the depolymerization of the microtubules, the spindle shrinks and the spindle fibers shorten considerably, changing the spindle’s morphology (Figs. 3 and 11). On the addition of 2 μM Ca^{2+}, the astral fibers curl and shorten inward toward the centrosome or centrosphere. The half-spindle fibers shorten, with the chromosome-to-pole distance frequently decreasing by 40–50% of its initial length. The spindle shape becomes more triangular. The initial rate or shortening of metaphase half-spindles is typically 10 μm/minute for 2 μM Ca^{2+} and 100 μm/min for Ca^{2+} concentrations of 10 μM and above (Salmon and Segall, 1979). If only one pole of a metaphase spindle is anchored to the slide, this calcium-induced spindle-fiber shortening produces a mass movement of the chromosomes toward the attached pole (Fig. 11). This effect is similar to the experimentally induced movement of chromosomes toward an anchored pole observed by Inoué and co-workers in living meiotic metaphase-arrested oocytes of Chaetopterus (Inoué and Ritter, 1975; Salmon, 1975, 1976). Although in our initial report of these observations, our calcium buffers contained ATP, we have since then been unable to demonstrate a requirement for ATP hydrolysis for the

![Fig. 11. Shortening of an isolated metaphase spindle from L. variegatus induced by addition of 2 μM Ca^{2+}. (As mentioned in the text, asters can be easily sheared off metaphase spindles during isolation.) The spindle remains anchored to the slide near the upper pole. Spindle-fiber shortening results in the chromosomes being transported toward the anchored pole, but half-spindle shortening is symmetrical. This spindle, initially in an EGTA buffer without glycerol, was perfused with an EGTA buffer containing 2 μM Ca^{2+} and 1 mM MgATP (Salmon and Segall, 1980) at time (in minutes) set = 0. The chromosomes move toward the upper anchored pole at an initial rate of 6 μm/minute. The presence of ATP induces decondensation of the chromosomes seen at 3 minutes.](image-url)
Fig. 12. Isolated metaphase spindle from *L. variegatus* augmented with purified brain MTP for 30 minutes at 23°C as viewed with polarization microscopy at zero compensation. Over 30 minutes the maximum spindle BR increased from 3.2 nm to 6.8 nm, with the location of maximum BR moving from the center of each half-spindle to the metaphase plate region. The MTP, purified from porcine brain (Salmon and Segall, 1980), was at 1.35 mg/ml in reassembly buffer (Table II). The spindles were pretreated in EGTA-glycerol storage buffer with 10 mM EDTA at pH 6.8 for 1 hour before perfusion with the reassembly buffer containing MTP. Perfusion of other EDTA-treated spindles with reassembly buffer without tubulin resulted in the disappearance of spindle BR within about 10 minutes. Bar = 10 μm; magnification, ×1200.

calcium-induced microtubule depolymerization on spindle-fiber shortening (Salmon and Segall, 1979).

Note that the effects of calcium on *L. variegatus* isolated spindles are not general effects of divalent cations (Salmon and Segall, 1980), and they are distinctly different from the general ionic effects on the hydration of isolated mitotic apparatuses prepared by the hexylene glycol method (Cohen, 1968). The swelling and shrinking in low- and high-ionic-strength buffers of the mitotic apparatuses isolated with hexylene glycol may result from osmotic effects on the
volume of the membrane vesicles that are preserved in mitotic apparatuses isolated with hexylene glycol from sea urchins.

Isolated spindles transferred into EGTA buffer or microtubule-reassembly buffer are relatively stable to the absence of an exogenous tubulin pool, cooling to 4°C, pressurization to 8000 psi, or 100 μM colchicine—agents that depolymerize the mitotic spindles rapidly (within 1–2 minutes) in the living cell. However, if the spindles in EGTA-glycerol storage buffer are treated for 1 hour with 10 mM EDTA, then the stability of the spindle microtubules when transferred to an EGTA or reassembly buffer is directly dependent on the assembly characteristics of an exogenous tubulin pool (Salmon and Segall, 1980; Salmon et al., 1980). The EDTA-treated spindles are similar to spindles in vivo in their spindle-fiber BR, their morphology, and their lability to cold and pressure. The isolated spindles’ calcium lability is not inhibited by pretreatment with EDTA.

Both EGTA- and EDTA-treated spindles will incorporate purified brain MTP (Fig. 12). The central-spindle BR increases from 3 to 6 nm with 1.5 mg/ml exogenous MTP, but little interpolar elongation occurs. Isolated spindles augmented with calcium-insensitive 6S brain tubulin lose their lability to micromolar calcium (Salmon et al., 1980). Preliminary results indicate that addition of 100 μM colchicine to the MTP-reassembly medium will block augmentation of spindle BR in vitro, but does not promote net disassembly as colchicine does in vivo (E. D. Salmon, unpublished observations). The effects of colchicine on isolated spindles are similar to the effects of colchicine on the self-assembly of brain MTP in vitro, but not to the effects of colchicine on spindle-microtubule assembly in living cells (Inoué, 1952).

VII. Future Considerations

Current theories of mitosis are all based on proposed structural-functional properties of spindle-fiber microtubules, but no theory has yet provided a completely satisfactory explanation of the mitotic process. Microtubules are undoubtedly key functional components for moving chromosomes. The EGTA-isolated spindles have permitted us to focus attention on the structure and function of the spindle-fiber microtubules and have suggested that two additional components of the mitotic apparatus may be important: (1) the fine filamentous network that enmeshes the microtubules of EGTA-isolated spindles; and (2) the membranes of the SER, which are totally absent in EGTA-isolated spindles. Our work with EGTA-isolated spindles from L. variegatus suggests that spindle shortening can be induced in a manner that transmits forces sufficient to displace the chromosomes simply by adding calcium. No ATP hydrolysis appears to be necessary. Perhaps the meshwork of 3- to 4-nm fine filaments associated with
and cross-linking the microtubules acts as a contractile gel, or induces spindle shortening by a mechanism similar to the action of calcium-activated contractile fine filaments in the spasmone (Amos, 1975). Interestingly, chromosome movement continues to a limited extent in anaphase mitotic apparatuses isolated by Sakai et al. (1979) with an EGTA–glycerol lysis buffer that contains no detergent. Silver et al. (1980), by using a low-ionic-strength EGTA lysis buffer similar to the one described here but without detergent, have recently obtained from S. purpuratus isolated mitotic apparatuses that retain the endogenous membrane components and that actively sequester calcium ions. It would be of interest to compare closely spindles and mitotic apparatuses isolated in buffers that are similar except for the presence or absence of detergent. However, I have not yet been successful in isolating mitotic apparatuses from L. variegatus or Arbacia with Silver’s method; this also may be a problem of species specificity.

The EGTA-isolated spindles provide a simplified preparation for analyzing spindle structural and functional characteristics, including microtubule-assembly mechanisms and polarity; tubulin flux; possible microtubule-associated proteins (MAPs, calmodulin, dynein, for example); the mechanism of calcium depolymerization; the action of other possible physiological regulators of microtubule assembly such as pH, phosphorylation, sulphydryl oxidation-reduction; and action of drugs that affect spindle-microtubule assembly in vivo, such as colchicine. The role of dynein or myosin in the generation of motility can be tested in ‘add-back’ experiments. Future attention should also be given to the structure and composition of the centrosome-centrosphere complex, the ribosome-like particles, and the spindle remnant that persists after the microtubules are depolymerized.

We are currently studying the physiological properties of isolated spindles that have been returned to buffers containing estimated normal physiological concentrations of K⁺, Cl⁻, Mg²⁺, glycine, glutamate, and soluble protein. Mg²⁺ concentrations above 0.5 mM clearly stabilize the spindle microtubules, and studies of isolated spindles in buffers with high Mg²⁺ concentrations may be misleading. Although raising the ionic strength of the EGTA buffer makes the spindle microtubules more labile and lifelike, lysis of cells in a high-ionic-strength physiological buffer without polymerizable exogenous tubulin does not yield isolated spindles. Purified brain tubulin stabilizes microtubules in isolated spindles, but can modify their calcium lability (Salmon et al., 1980). It is not clear whether this effect is a result of the source of tubulin (egg tubulin might be better) or to the tubulin-purification process (a calcium-binding microtubule-associated protein may be lost during purification). Kuriyama (1977) has developed a method of purifying egg tubulin from sea urchins, but enormous quantities of eggs are needed to yield a useful quantity of tubulin. The best approach currently seems to be to use the low-ionic-strength EGTA lysis buffer for isolating and storing the spindles, and then for experiments to return the spindles to a
physiological buffer that contains egg tubulin and that more nearly duplicates the composition of egg cytoplasm.

ACKNOWLEDGMENTS

I would like to thank Tim Otter for his critical review of the manuscript and Nancy Salmon for her usual outstanding editorial assistance. I also appreciate the aid of Mike Spillane and Wilma Hanton with the electron microscopy.

This work was supported by grants from the National Institutes of Health (GM 24364) and the National Science Foundation (76–09654 and 77–07113).

REFERENCES


5. MITOTIC SPINDLES ISOLATED FROM SEA URCHIN EGGS