

# "Spiral Asters" and Cytoplasmic Rotation in Sea Urchin Eggs: Induction in *Strongylocentrotus purpuratus* Eggs by Elevated Temperature

THOMAS E. SCHROEDER and DAVID E. BATTAGLIA

Friday Harbor Laboratories, University of Washington, Friday Harbor, Washington 98250. D. E. Battaglia's present address is Department of Biological Structure, University of Washington, Seattle, Washington 98195

**ABSTRACT** "Spiral asters" composed of swirls of subcortical microtubules were recently described in fertilized eggs of the sea urchin *Strongylocentrotus purpuratus*. In our study, these structures did not occur at culture temperatures below 16°C. When the culture temperature was elevated, however, "spiral asters" routinely appeared during a susceptible period before mitotic prophase when the sperm aster-diaster normally exists. A massive and protracted rotation of the cytoplasm (excluding an immobile cortex and perinuclear region) began within 1 min of exposure to elevated temperature. Fibrils of the "spiral aster" could be seen within this rotating mass even by bright-field microscopy. The identity of microtubules in these structures was confirmed by indirect immunofluorescence microscopy.

A mechanistic association between "spiral aster" formation and cytoplasmic rotation was indicated by the simultaneous inhibitory effects of microtubule and dynein poisons. Inhibitors of microfilaments, however, had no effect. We infer that elevated temperature induces unique changes in the microtubules of the pre-prophase sperm aster-diaster, resulting in cytoplasmic rotation and the spiral configuration of microtubules.

Comparative cytological evidence supports the idea that "spiral asters" do not normally occur in fertilized sea urchin eggs. Biogeographic evidence for *S. purpuratus* indicates that fertilization and development naturally occur below 15°C, hence "spiral asters" in eggs of this species should be regarded as abnormalities induced in the laboratory by unnaturally elevated temperatures.

The organization and functions of premitotic and mitotic asters have long been a focus of study in fertilized sea urchin eggs. Recently, "spiral asters" were described in eggs of *Strongylocentrotus purpuratus* during a relatively brief period midway between fertilization and first cleavage. They were initially observed in resin-embedded whole-mounts and sections by light microscopy (10). Subsequent electron microscopy and indirect immunofluorescence microscopy established that these "spiral asters" are composed of swirls of microtubules in a subcortical zone 10–15  $\mu\text{m}$  from the egg surface (11, 12). Nevertheless, the existence of "spiral asters" in *S. purpuratus* eggs does not accord with previous descriptions of other species of sea urchin eggs, some of which are much more amenable to in vivo observation and have a longer tradition

of cytological study. Ordinarily, the period midway between fertilization and first cleavage is characterized by the expanding sperm aster and its direct descendant, the pre-prophase diaster, both of which contain radial arrays of microtubules.

Two incidental observations led us to suspect that "spiral asters" might not be natural features of *S. purpuratus* development but, instead, might be induced by experimental conditions that alter microtubules. First, in fertilized eggs treated with the tubulin-polymerizing drug taxol, a subcortical shell-like layer of relatively granule-free or "clear" cytoplasm can be seen by light microscopy (29; Schroeder, T. E., unpublished observations). In addition, even untreated eggs develop similar "clear" zones when allowed to remain too long at room temperature on a microscope. Here we examine the hypoth-

esis that "spiral asters" occur within such "clear" zones induced by unnaturally elevated temperature and thus are laboratory artifacts.

## MATERIALS AND METHODS

Adult sea urchins (*S. purpuratus*) were collected in early winter from Clallam Bay, Washington (Strait of Juan de Fuca) and stored in submerged cages suspended from the dock at Friday Harbor Laboratories until they were used. They were periodically fed fronds of *Nereocystis luetkeana*. Gamete collection (by injection with isotonic KCl), washing, insemination, and culturing were carried out at 10–12°C, and care was taken to prevent heat-shocks at any stage.

When fertilization envelopes were removed, two methods were employed. A conventional procedure (30) using 1 M urea, passage through nylon mesh, and washing in calcium-free seawater (32) produced fertilized eggs devoid of all coats including hyaline layers. Alternatively, the use of neutralized 0.1% mercaptoethylgluconamide in seawater and passage through nylon mesh (11) yielded fertilized eggs with intact hyaline layers. In other experiments, unaltered fertilized eggs were cultured in filtered seawater so that all coats were left intact.

For microscopic observations of living cells, several eggs were transferred gently but swiftly to a temperature-controlled microscope stage at various times beginning ~30 min after fertilization. Overlying coverslips were supported by small clay "feet" to prevent physical compression of the eggs. Eggs were initially mounted onto a cool stage at a temperature matching that of the culture, and then the temperature was selectively changed. A thermoelectric cooling stage (6) or a flowing-water culture slide (34) was used to control the temperature of eggs during microscopic observations. Temperature could be shifted by 1–2°C increments at will; shifts of up to 10°C were achieved within 15–30 s.

To augment photomicrographic records, video sequences were obtained with an accessory video camera (model 65; Dage-MTI Inc., Michigan City, IN) and a time-lapse video recorder (model 2051; Gyr Products, Anaheim, CA). Intracellular motion is illustrated here in time-exposure photographs of the video monitor's image using 35-mm film (Panatomic X; Eastman Kodak Co., Rochester, NY); for example, 48-s intervals of intracellular motion were obtained by 8-s time-exposures of a time-lapse sequence while it played at six times normal speed. Instantaneous images were photographed from still-frames of the video record.

For experiments requiring substantial numbers of eggs, small dishes containing 5 ml of egg cultures (initially grown at 10–12°C) were placed at room temperature (18–23°C) and allowed to equilibrate; for controls, aliquots of the same culture were left in equivalent dishes at 10–12°C. Glutaraldehyde-fixed, resin-embedded whole-mounts were prepared by the method of Harris (10). Tubulin-containing structures were identified in eggs (lacking fertilization envelopes and attached to polylysine-coated coverslips) by the indirect immunofluorescence method, as previously described (24), using a monoclonal antibody against alpha-tubulin (3). Attachment of the eggs to polylysine-coated coverslips before fixation in cold methanol was conducted at the experimental temperature; for controls, the attachment was performed in a cold room at 10°C in order to prevent heat shock during this step.

## RESULTS

### Cytoplasmic Rotation

The cytoplasm of normal fertilized *S. purpuratus* eggs cultured at or below 12°C exhibited a pattern of radiating astral rays belonging to the sperm aster-diaster system during the period from 30 to 80 min postinsemination. The radiating astral rays were barely detected in these semi-opaque eggs by bright-field microscopy (with reduced condenser numerical aperture) or by differential interference contrast microscopy (Nomarski) as linear patterns among the cytoplasmic particles. Instantaneous images of eggs during this period are illustrated in Figs. 1*a* (a bright-field image from a video monitor) and 2*a* (a Nomarski image of a fixed egg). Time-lapse recordings and 48-s time-exposures (Fig. 1*b*) revealed no organized cytoplasmic motion at low temperatures. On the contrary, cytoplasmic particles remained virtually stationary.

At high temperatures (18–23°C), the cytoplasm of eggs at the same stage exhibited a pronounced rotational translation that persisted for up to 30 min. When the axis of rotation in

an egg was perpendicular or oblique to the optical axis, particles appeared to move laterally. When the axes coincided, however, it became clear that a large mass of cytoplasm was rotating as a unit. Cytoplasmic rotation was clockwise in some eggs and counterclockwise in others. This behavior was consistently seen in all eggs from several batches.

Instantaneous images of eggs exhibiting cytoplasmic rotation are shown in Figs. 1*c* and 2*b*. Rotation of the cytoplasm is dynamically conveyed in a 48-s time-exposure image (Fig. 1*d*), as indicated by the zone of blurred arcs formed by moving particles; particles in nonrotating regions are not blurred. In Fig. 1*d*, the moving cytoplasm rotated clockwise through an arc of 5 degrees (i.e., at a rate of 6 degrees per min).

Cytoplasmic rotation at elevated temperatures consistently established four concentric cytoplasmic zones within an egg (Fig. 3). Zones A–C were 8, 10, and 6  $\mu\text{m}$  thick, respectively, and surrounded a perinuclear core (zone D) ~32  $\mu\text{m}$  in diameter. Rotation was confined to the middle two zones (zones B and C), the outer of which (zone B) appears "clear" in instantaneous images (Figs. 1*c* and 2*b*). The outermost and innermost zones (zones A and D) did not participate in cytoplasmic rotation. The immobile cortex (zone A) of experimental eggs usually appeared "darker" and more radially organized by bright-field optics (Fig. 1, *c* and *d*) than it did in control eggs at lower temperatures (Fig. 1, *a* and *b*).

The angular rate of cytoplasmic rotation varied somewhat between eggs. Rotation rates of 3–11 degrees of arc per min have been recorded. In lengthy video records, the cytoplasm rotated through nearly 180 degrees of arc. The rate of rotation was identical for particles throughout the moving mass (zones B and C); that is, there was no obvious gradient of motion along a radius, except of course at the two abrupt shear zones between zones A and B and between zones C and D (Fig. 3).

There were no discernible differences in the rates or other details of cytoplasmic rotation between eggs with intact fertilization envelopes and hyaline layers and eggs that were denuded of one or both of these layers with urea or mercaptoethylgluconamide. Likewise, eggs cultured in natural or calcium-free seawater behaved indistinguishably.

### "Spiral Asters"

At high magnification, curving linear images are visible within zone B in eggs undergoing cytoplasmic rotation. These structures were particularly evident in resin-embedded whole-mounts, confirming previous observations (10), and suggesting the presence of microtubules or microtubule bundles in "spiral aster" configurations. In living eggs, these curved lines were related to the direction of cytoplasmic rotation as shown in Fig. 3; that is, their outer limbs "trailed" the rotation.

To visualize the distribution of tubulin-containing structures, fertilized eggs were stained with antitubulin antibody by the indirect immunofluorescence technique. Control eggs (Fig. 4*a*) were grown at 12°C and were prevented from experiencing elevated temperatures even during attachment to polylysine-coated coverslips before fixation. Antitubulin staining faintly revealed straight elements radiating from a pair of bright dots (putative centrosomes) at opposite sides of the central nucleus (Fig. 4*a*). The straight elements presumably represent microtubules of the astral rays of the normal diaster. Spiral arrays of microtubules were never seen in control eggs.

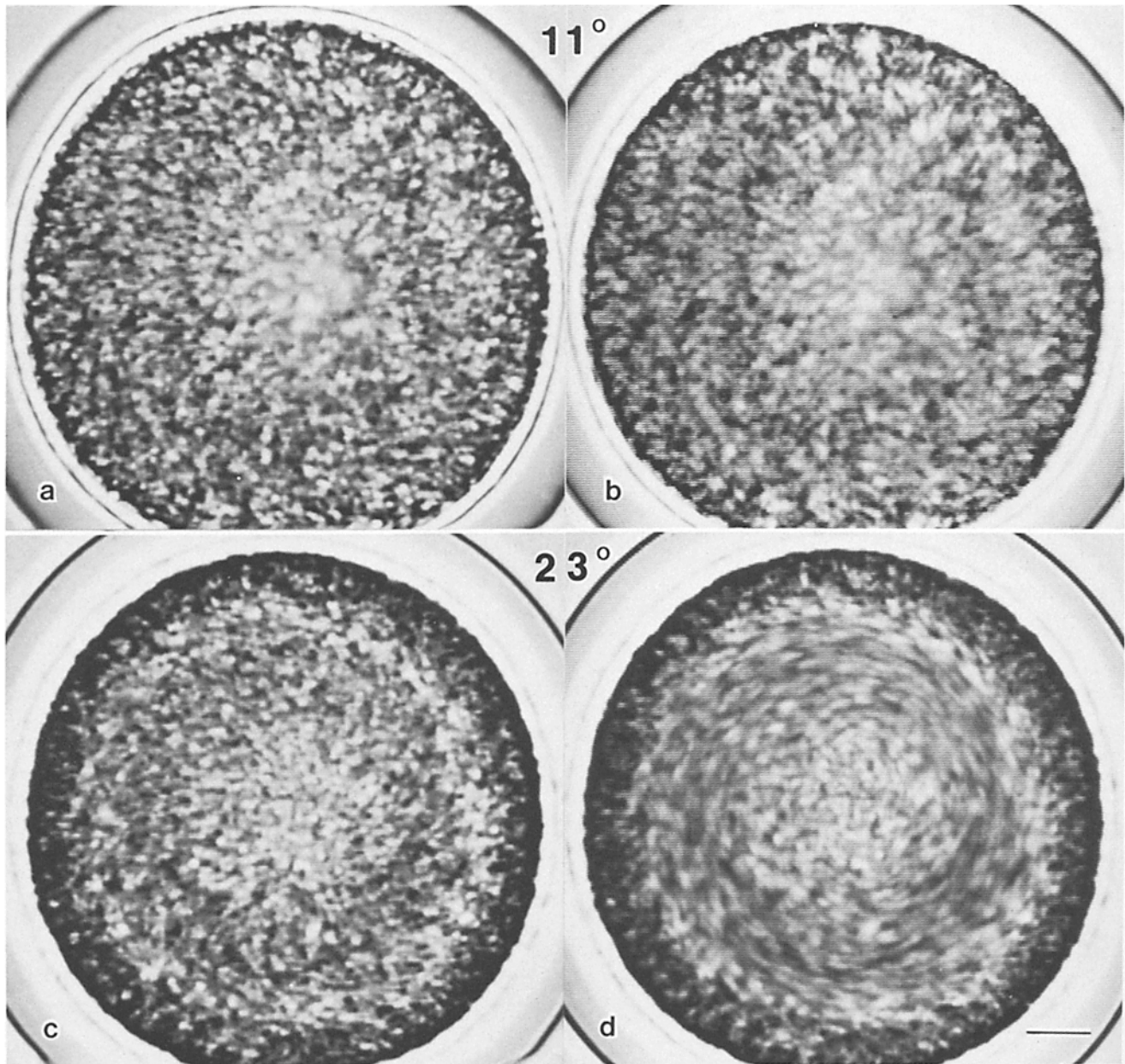


FIGURE 1 Photographs from a video record showing cytoplasmic rotation in a fertilized *S. purpuratus* egg when the culture temperature was elevated from 11°C (a and b) to 23°C (c and d). The focal level changed slightly when the temperature was elevated at 65 min postinsemination. a and c are still-frames of the video record at 64 and 72 min postinsemination, respectively. b and d are time-exposures showing cytoplasmic rotation during the 48 s following the still-frames of a and c, respectively. At 11°C, only a small amount of random motion of cytoplasmic particles can be detected in b, as compared with a. At 23°C, a large mass of cytoplasm rotates, as indicated by the blurred images of cytoplasmic particles in d; in this case, the cytoplasm rotated clockwise through 5 degrees of arc (6 degrees per min). A central perinuclear mass and the cortical cytoplasm do not participate in the rotation of the intervening cytoplasmic mass, as indicated by the lack of blurred images in these regions. This egg was raised in seawater with its fertilization envelope and hyaline layer still intact. Bar, 10  $\mu$ m. Bright-field optics ( $\times$  1,000).

Experimental eggs were allowed to warm up gradually to 22°C for 10 min before antibody staining. Such eggs consistently contained a prominent swirled or spiral array of brightly staining structures, presumably representing microtubules of a "spiral aster" (Fig. 4b). The outermost portions of these spiral arrays were aligned nearly circumferentially (corresponding to zone B, Fig. 3), whereas inner portions showed reduced numbers of microtubules coursing more radially toward the center of the egg (zone C). Each egg contained a single spiral array organized around a major axis. Spiral arrays

curved in apparently opposite directions in different eggs (which could equally be interpreted as intrinsically opposite spirals or of opposite views along axes of similar spirals).

In general, our indirect immunofluorescence images of microtubules in these eggs exhibited higher background fluorescence in the cytoplasm than those obtained by Harris et al. (11, 13). This background may represent unpolymerized tubulin in the cytoplasm, since this should be detectable by antibody staining. We never observed the starkly contrasted images or seemingly aggregated fibrils previously pictured

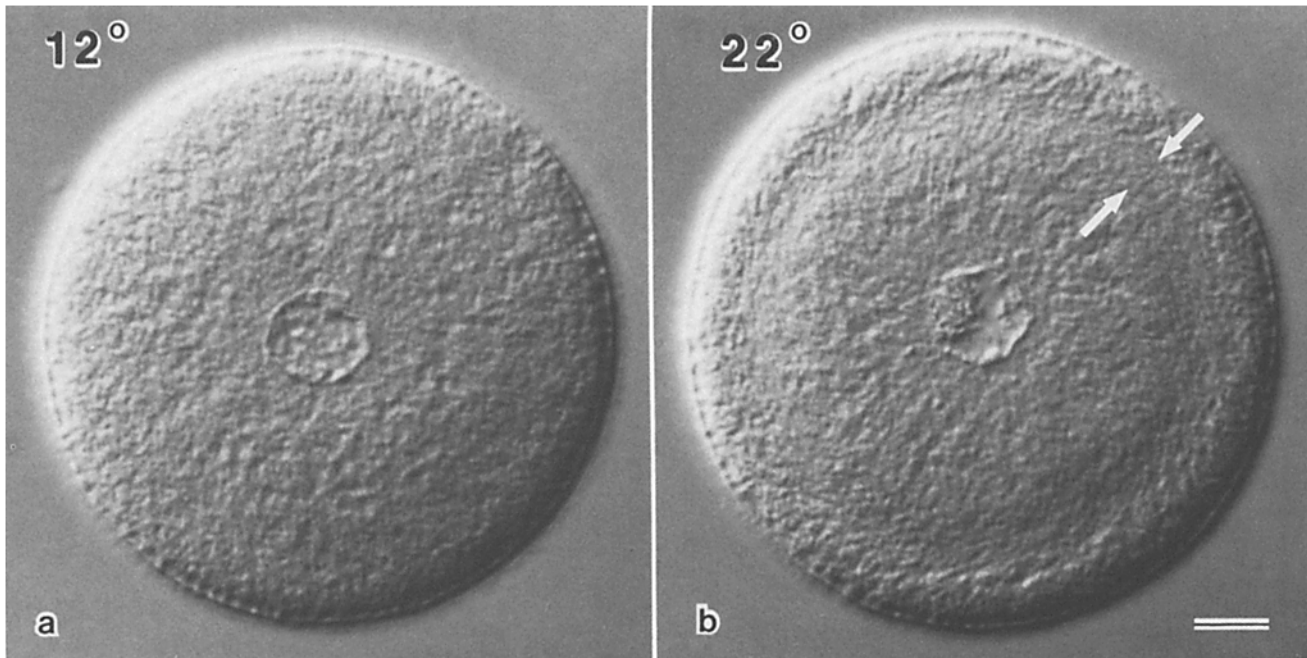


FIGURE 2 Resin-embedded whole-mounts of eggs from a single culture that were fixed in glutaraldehyde 52 min postinsemination. The egg in *a* was cultured continuously at 12°C. The egg in *b* was raised at 12°C for 47 min postinsemination and then allowed to warm up to 22°C over a 5-min period; it exhibits a unique subcortical zone (arrows) which, at higher magnification, contains linear fibrils of the "spiral aster." These eggs were denuded with mercaptoethylgluconamide and were raised in seawater. Bar, 10  $\mu$ m. Nomarski optics ( $\times$  1,000).

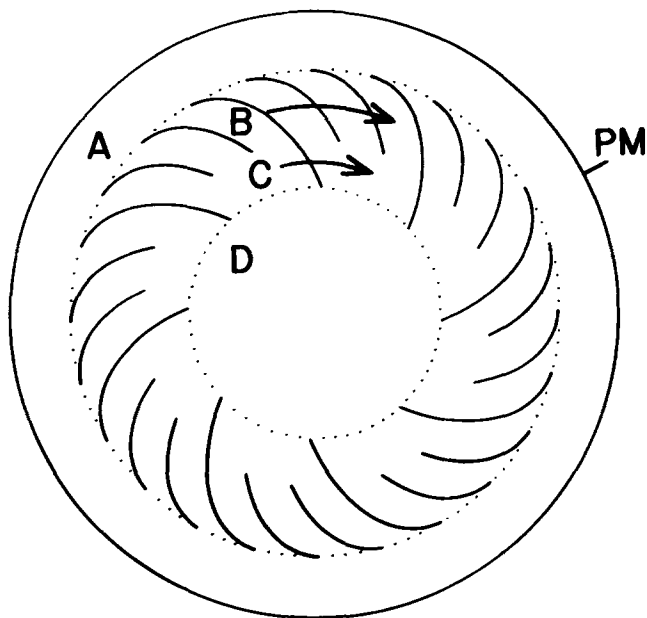


FIGURE 3 Diagram of the cytoplasmic zonation that is set up when eggs are exposed to elevated temperature. Zones A and D remain stationary. Zones B and C, containing microtubules of the "spiral aster," undergo cytoplasmic rotation at a constant rate, as indicated by arrows. Two shear-zones between rotating and stationary portions of the cytoplasm are indicated by dotted lines. The microtubules in zone B are nearly circumferentially aligned within a relatively granule-free "clear zone," as seen in living eggs with Nomarski or bright-field optics. Zone C contains fewer microtubules and they are more radially aligned. *PM*, plasma membrane.

(12), and assume that these different results have their origin in technical differences that have not yet been identified.

### Details of the Temperature Effect

Since cytoplasmic rotation and "spiral aster" formation seem to be coordinately induced by elevated temperature, we attempted (*a*) to define the threshold temperature at which rotation begins, (*b*) to determine if the effect is reversible by lowering the temperature, and (*c*) to discover if the rate of rotation is temperature dependent. These experiments were carried out by time-lapse video recordings of the behavior of individual eggs during step-wise heating and cooling on a temperature-controlled microscope stage. Rates of particle rotation were plotted by marking directly on the screen of the video monitor while replaying recorded sequences.

Despite some variation between individual eggs, as the temperature was raised, cytoplasmic rotation usually began at  $\sim$ 16°C and always occurred at 18°C. Rotation was plainly evident within 1 min, and a plateau rate of rotation was achieved by 2 min. In any individual egg, once rotation had begun and reached a plateau rate, it did not noticeably increase further as the temperature increased from 16° to 23°C. Upon cooling, cytoplasmic rotation ceased abruptly, in some cases, at 15°C and always by 10°C. Despite the cessation of cytoplasmic rotation, "spiral asters" remained microscopically evident within zone B for at least several minutes. Occasionally, rotation ceased even without cooling, but this could be attributed to the progressive stage of development, since eggs ordinarily failed to respond to elevated temperature after  $\sim$ 80 min postinsemination (i.e., the beginning of prophase).

### Effects of Inhibitory Drugs

To explore the mechanistic basis of cytoplasmic rotation (and thus of "spiral aster" formation), we examined the effects of drugs known to inhibit aspects of microtubule or microfilament systems in eggs. Accordingly, drugs were added to 1

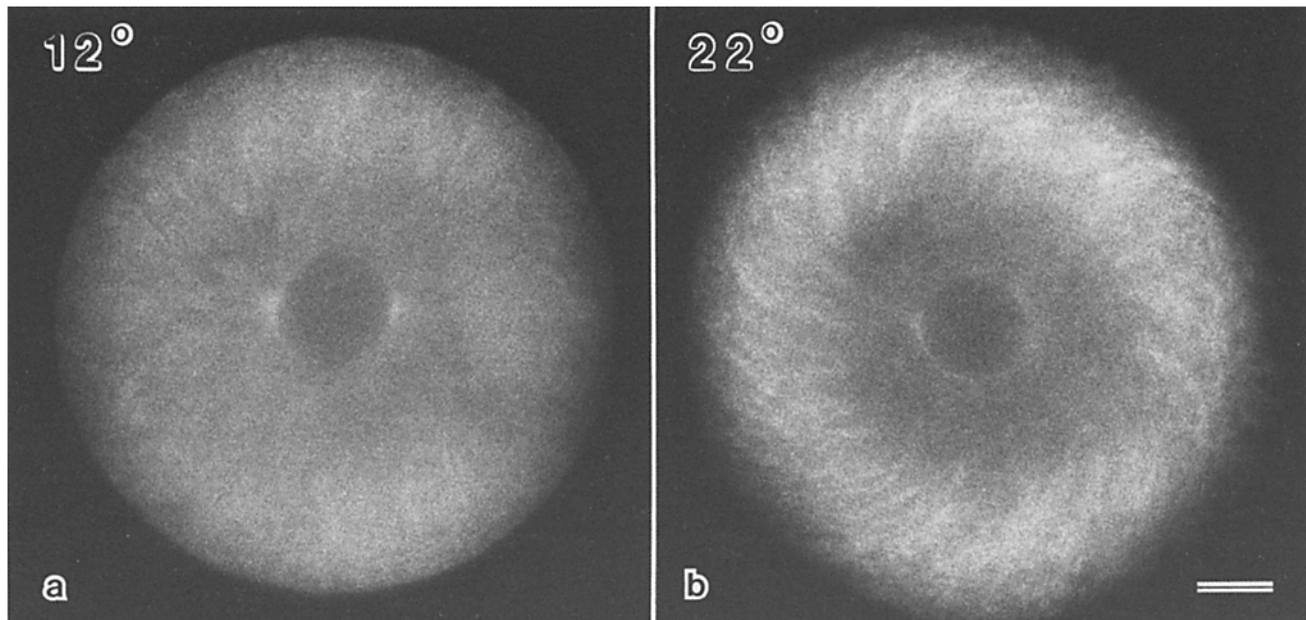


FIGURE 4 Indirect immunofluorescence micrographs of eggs stained with antitubulin antibody showing the effect of elevated temperature in inducing "spiral asters." Microtubules and microtubule bundles are indicated by bright lines; unpolymerized cytoplasmic tubulin may be indicated by background levels of fluorescence. Both eggs were taken from the same culture and were fixed at 64 min postinsemination. The egg in *a* was raised continuously at 12°C; the microtubules are inconspicuous straight lines radiating from two perinuclear foci (presumably the centrosomes) in the pattern that is typical of the pre-prophase diaster. The egg in *b* was allowed to warm up to 22°C over 10 min beginning at 54 min postinsemination. A prominent "spiral aster" of curvilinear bundles of microtubules is evident; the perinuclear zone is relatively deficient of staining, although the putative centrosomes are still evident as dots adjacent to the nucleus. These eggs were denuded in urea and were raised in calcium-free seawater. Bar, 10  $\mu\text{m}$ .  $\times 1,000$ .

ml of egg suspension at  $\sim 45$  min postinsemination after rotation had been induced by warming to room temperature for 5 min. Cytoplasmic rotation was monitored by time-lapse video-microscopy for the next 10–15 min. The microfilament-inhibiting drugs cytochalasin B, cytochalasin D, and dihydrocytochalasin B (10  $\mu\text{g}/\text{ml}$ ) had no discernible effect; rotation proceeded as in untreated controls at room temperature. The microtubule-depolymerizing drugs colchicine (5 mM) and nocodazole (10  $\mu\text{M}$ ) arrested cytoplasmic rotation within 2 min. Erythrohydroxynonyladenine-HCl (5  $\mu\text{M}$ ), a putative inhibitor of dynein (4), also rapidly inhibited cytoplasmic rotation.

### Developmental Consequences

In two batches of fertilized eggs cultured nominally at 10°C, aliquots from each were exposed transiently to room temperature (22°C) for 10 min to induce cytoplasmic rotation, whereas control aliquots were not warmed. Development of both sets of experimentals and controls was followed to the end of gastrulation. Except for a slight acceleration of the very early cleavage divisions in the experimentals, no differences could be discerned between them and the controls in terms of the geometry or schedule of embryonic stages, including the 16-cell embryo, blastula swimming and hatching, or the onset and completion of archenteron invagination.

## DISCUSSION

### Do "Spiral Asters" Occur Normally?

"Spiral asters" composed of microtubules were originally described in eggs of *S. purpuratus* and were interpreted, with

certain reservations, to be a normal cytological feature of the period between fertilization and first cleavage (10–12). In this study, we have reproduced "spiral asters" in the same species and have discovered that they are associated with a prominent cytoplasmic rotation. Both phenomena are consistently seen at culture temperatures above 16°C, whereas they never occur when the culture temperatures are carefully controlled at 12°C or lower.

The proposition that "spiral asters" are normal or widespread constituents of developing sea urchin eggs is inconsistent with a respected body of comparative cytology. Observations of "spiral asters" are conspicuously absent from the thorough cytological studies in the earlier literature (e.g., 5, 8, 9, 13, 15, 35, 36) and have not been recorded in more modern light microscopic observations of transparent eggs (e.g., 22, 28) or by electron microscopy (18). Moreover, recent antitubulin immunofluorescence studies of *Lytechinus variegatus* and *Arbacia punctulata* eggs have specifically failed to detect "spiral asters" (2). Nevertheless, we find that they are readily detectable even by bright-field microscopy when *S. purpuratus* eggs are treated as described above.

Despite this negative evidence in normal eggs, structures reminiscent of "spiral asters" have been reported when eggs were subjected to such overtly unnatural treatments as hypertonicity (20), traumatic shaking (26), parthenogenetic activation (7, 19, 25), and the drugs stypoldione (21) and taxol (29; Schroeder, T. E., unpublished observations). Thus, "spiral asters" (and perhaps cytoplasmic rotation) may be induced by several conditions that are abnormal to sea urchin eggs.

It is widely accepted, as stated by Hinegardner (14), that *S. purpuratus* "from central California and farther north begins to show developmental abnormalities above 15°C." Indeed, it

is likely that fertilized eggs of *S. purpuratus* in nature rarely encounter environmental temperatures above 15°C, at which "spiral asters" and cytoplasmic rotation occur in the laboratory. Throughout its range in the northeastern Pacific Ocean from Isla Cedros, Mexico to the eastern margin of the Gulf of Alaska, *S. purpuratus* spawns naturally mostly in winter (January to March) when the temperature, reported from depths of 10 m, is typically 7.5–15°C (1).

We interpret all of this evidence to signify that "spiral asters" and cytoplasmic rotation are artifacts attributed to exposure to unnaturally elevated temperatures. We do not know if this response is limited to *S. purpuratus* or if it occurs in other cold-water species. Nevertheless, it underlines the importance of preventing laboratory cultures of *S. purpuratus* eggs from experiencing temperatures in the "room temperature" range, even for as briefly as 1 min.

### *The Normal Pre-Prophase Aster Cycle*

While concluding that "spiral asters" are artifacts, we wish to affirm our interpretation of the normal aster cycles (that is, assembly-disassembly of astral rays) in sea urchin eggs. Classical descriptions indicate that each mitotic cycle is associated with one discrete aster cycle, but that the pre-prophase period after fertilization exhibits one additional aster cycle that produces the sperm aster-diaster.

We concur with the classical interpretation of events in normal fertilized sea urchin eggs, namely that the sperm aster acquires a progressively bipolar organization as the centrosome splits and evolves directly into a diaster within the same cycle of aster assembly-disassembly. Accordingly, the sperm aster-diaster comprises the first aster cycle, persists from fertilization through the time of pronuclear fusion to a stage ambiguously known as the "pause" (coinciding roughly with the equally ambiguous "streak" stage), and functions to promote pronuclear fusion and to establish and maintain a centralized zygote nucleus (8, 28, 35). Thereafter, rays of the sperm aster-diaster fade away, to be replaced gradually by the "amphiaster" whose small asters appear during prophase shortly before nuclear envelope breakdown. These new asters constitute the early mitotic asters (the second aster cycle). Rays of the mitotic asters elongate from prophase through telophase and even persist well beyond the end of cleavage when the blastomeres are technically in interphase. Thereafter, these mitotic asters fade away before the appearance of the mitotic asters of the second division (the third aster cycle).

Recently, more complicated schemes of the aster cycles in sea urchin eggs identify "spiral asters" and other "interphase asters" as products of supernumerary aster cycles (11, 28) that are not described in the classical studies. In part, some of these discrepancies can be attributed to disadvantageous properties of the much-used *S. purpuratus* egg, including its natural opacity and its susceptibility to form "spiral asters" at elevated temperatures.

### *Mechanism of "Spiral Aster" Formation and Cytoplasmic Rotation*

Confirming Harris et al. (12) we find that "spiral asters" and cytoplasmic rotation can only be induced from ~30 min to ~80 min postinsemination and not during mitosis. Since this period of susceptibility coincides with the pre-prophase period during which the diaster is present, we think that "spiral asters" are either direct elaborations of diasters or are

products of a physiological condition that uniquely occurs during this phase.

The staining patterns by antitubulin immunofluorescence suggest that eggs with "spiral asters" possess a greater number and/or length of microtubules than exists in control eggs. These microtubules seem to predominate in zone B and often appear depleted in zone C. Our present data cannot distinguish whether "spiral asters" arise through a proliferation of new microtubules or by elongation or rearrangement of preexisting ones. Stephens (33) demonstrated a potentially related stimulation of microtubule assembly on fertilized eggs of *S. droebachiensis*, a congener of *S. purpuratus*. Warming during a restricted period before prophase caused an increase in the intracellular pool of tubulin that was competent to polymerize. The sensitive period appears to coincide with the phase of susceptibility to "spiral aster" formation and cytoplasmic rotation in *S. purpuratus* eggs.

Our experiments with cytochalasins do not support an involvement of actin microfilaments in "spiral aster" formation or cytoplasmic rotation. Nevertheless, elevated temperature may still cause important changes in the microfilament system of the cortex since we observed that it becomes thicker and more radially organized at room temperature (compare *a* and *c* in Fig. 1). Furthermore, in another species, elevated temperature causes egg microvilli to elongate excessively (17). Both of these observations are possibly explained by alterations induced in the complexes of polymerized actin and fascin in the cortex that normally support the microvilli (23, 31). Since the association of actin and fascin is temperature-sensitive *in vitro* (16), elevating the temperature of intact eggs may enhance their complexing in the cortex *in vivo*.

Since "spiral aster" formation and cytoplasmic rotation are coordinately induced, we assume that they are mechanistically linked. Our experiments with microtubule inhibitors (colchicine and nocodazole) indicate that cytoplasmic rotation requires the existence of polymerized microtubules. The involvement of dynein, the mechano-enzyme associated with microtubules (27), is suggested by the inhibitory effect of erythrohydroxynonyladenine but remains inconclusive due to uncertainties about the specificity of this drug. Regardless of the molecular basis for the motive force, it seems likely that mechanical traction for cytoplasmic rotation occurs at the subcortical and/or perinuclear shear-zones (dotted lines, Fig. 3). Thus, even though "spiral asters" and cytoplasmic rotation may be artifacts, further study of the underlying causal mechanisms may help to elucidate the dynamics of the cytoskeleton in fertilized sea urchin eggs.

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