THE CONTRACTILE RING

II. Determining its Brief Existence, Volumetric Changes, and Vital Role in Cleaving *Arbacia* Eggs

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ABSTRACT

The first cleavage furrow in eggs of *Arbacia* (sea urchin) is accompanied by a uniform ring of aligned microfilaments, called the contractile ring. Individual contractile ring filaments measure 35–60 A and occasionally appear "hollow." The contractile ring exists from about 20 sec after anaphase to the end of furrowing activity, i.e., 6–7 min at 20°C. It is closely associated with the plasma membrane at all times, and is probably assembled there. It is about 8 μ wide and 0.2 μ thick throughout cleavage. Its volume decreases, however, suggesting a contraction-related disassembly of contractile ring filaments, rather than a sliding-filament mechanism in the strict sense. Cytochalasin B ($>10^{-6}$ M) arrests cleavage within 60 sec, by which time contractile ring filaments are no longer visible ultrastructurally. The furrow may be seen to recede within this time. Karyokinesis is unaffected. Simultaneous disruption of furrowing activity and of the contractile ring largely confirms the vital role of the contractile ring as the organelle of cell cleavage.

INTRODUCTION

Recently a discrete organelle called the "contractile ring" has gained acceptance as the agent responsible for the mechanical act of cytoplasmic cleavage in animal cells (see Schroeder, 1970 *c*). The contractile ring (CR) was originally postulated by Marsland and Landau (1954) and its existence was confirmed by electron microscopy (Schroeder, 1968). It has been described as a transitory structure composed of circumferentially aligned microfilaments (CR filaments) encircling the zone of cell constriction.

A working hypothesis for the role of the CR is that it exerts annular contractile forces upon the cell during division. Its precise mode of action remains unknown, although a current macromolecular model suggests that force-generation is achieved by concerted mutual sliding interactions between neighboring CR filaments. This model borrows heavily from the sliding-filament mechanism proposed for muscle contraction (Huxley and Hanson, 1954; Huxley, 1969; and others) even though the latter mechanism depends upon two dissimilar filament populations. The model further presupposes a mechanical association between the CR and the plasma membrane, which might be visualized as a counterpart of the intercalated disc in cardiac muscle tissue. Assuming that CR filaments are conserved during actual contraction, other consequences of such a simplified sliding-filament mechanism are either that lateral spacing between CR filaments decreases or that the total volume of the CR remains constant. These and other considerations concerning the assembly, force-generating properties, and disassembly of the

CR must be dealt with in striving for a full understanding of cytokinesis.

Eggs of *Arbacia punctulata* have been the subject of many investigations into the biology of cell division (Harvey, 1956; Chambers and Chambers, 1961). Much of this egg's fine morphology has been described recently by Anderson (1968). CR filaments in *Arbacia* eggs were specifically described by Goodenough et al. (1968) and Tilney and Marsland (1969).

The present article describes ultrastructural observations made before, during, and at the end of cell cleavage, in an endeavor to ascertain the precise time and manner of appearance and disappearance of the contractile ring relative to active contractile events. Changes in organization of contractile ring filaments during the course of cleavage have also been sought. Ultrastructural morphometry is employed to resolve whether the volume of the contractile ring remains constant, which would lend support to a simplified sliding-filament mechanism of action. The drug cytochalasin B is utilized to evaluate the functional role of contractile ring filaments in cleavage activity. In general, this investigation attempts to gather ultrastructural and experimental evidence about the role of contractile ring filaments in a familiar, normal cell type during cleavage. The evidence serves to modify existing postulates and is directed toward a theory of cytokinesis based upon the dynamics of the contractile ring. Some of the data reported here have appeared elsewhere in preliminary form (Schroeder, 1969).

MATERIALS AND METHODS

Gametes of *A. punctulata* from the vicinity of Woods Hole, Mass., were obtained by the electroshock method; removal of jelly coats and fertilization membranes was achieved by shaking 2.5 min after fertilization (Harvey, 1956). Demembranated eggs were rinsed twice in Shapiro's artificial calcium-free sea water (supplied by Marine Biological Laboratory, Woods Hole, Mass., see Harvey, 1956) and then allowed to develop in the same solution. This procedure was followed in order to avoid adhesion between eggs as well as between facing surfaces of blastomeres. Living eggs were maintained at 18–21°C.

Fixation for microscopy was achieved by depositing 1 or 2 drops of a concentrated egg suspension into about 5 ml of 2% glutaraldehyde in 83% sea water (natural) adjusted to pH 6.8 with NaOH for 15 min in polypropylene weighing bottles (Mallinckrodt Chemical Works, St. Louis, Mo.). This fixative was pipetted off and replaced by 0.5% osmium tetroxide in 75% sea water (unbuffered) for an additional 15 min. After rapid dehydration in ethanols and propylene oxide, eggs were infiltrated with Epon 812 *in vacuo* overnight and polymerized for 3 hr at 90°C. Since all procedures were conducted in the weighing bottles, eggs were never subjected to manipulation.

All microtomy was performed on preselected and precisely oriented eggs with a Sorvall MT-1 ultramicrotome (Ivan Sorvall, Inc., Norwalk, Conn.). Half-micron sections (Figs. 1 *a*, 2 *a*, 3 *a*, 4 *a*, and 8 *a*) were stained with methylene blue-azure II after Richardson et al. (1960). Thin sections (unsupported except by copper mesh grids) were stained in saturated uranyl acetate and lead citrate solutions (Venable and Coggeshall, 1965). Electron micrographs (Figs. 1 *b*, 2 *b*, 3 *b*, 4 *b-c*, 5, and 8 *b*) were obtained on a Siemens Elmiskop IA instrument operated at 80 kv with an objective aperture of 30 μ.

The morphometry expressed in Figs. 6 *a–b* depends upon definitions of: "width" (W_{cr}) as distance along the U from limb to limb as seen in meridional sections; "thickness" (T_{cr}) can be gauged in either meridional or equatorial sections, so long as they are precise; "minimum equatorial diameter" (D_{min}) measured across the cell's isthmus in a true meridional section, selected from a series of half-micron sections; S_n represents the center-to-center separation between nuclear elements, i.e. chromosomes, chromosomal vesicles, or nuclei. Values for W_{cr} (measured along the curvature) were obtained from electron microscope negatives at × 2500 viewed with a × 7 measuring magnifier. D_{min} values were obtained for the same cells from adjacent, or nearly so, half-micron sections by optical micrometry at × 400.

In approximating the volume of the CR, a simple relationship exists during the first third of cleavage (D = 75-50 μ), while the CR is roughly cylindrical, namely:

$$V_{cr} = W_{cr} \times D_{min} \times \pi \times T_{cr}\,.$$

Thereafter, the CR becomes increasingly curved (concave) which enhances the difference between D_{min} and other diameters, especially at the poleward "edges" of the CR. Approximate volume-width relationships during the second and final thirds of cleavage are expressed by the following equations, which are based upon "V"-shaped profiles of the CR:

$$V_{cr} = W_{cr}\,(D_{min} + 4) \times \pi \times T_{cr}\,; \qquad D_{min} = 49\text{-}25\mu$$

$$V_{cr} = W_{cr}\,(D_{min} + 8) \times \pi \times T_{cr}\,; \qquad D_{min} = 24\text{-}3\ \mu.$$

The theoretical curve $V_{cr} = k$ in Fig. 6 *a* is based upon these three equations.

In compiling data for Figs. 6 *a–b*, the following criteria were observed: (*a*) measurements were regarded as acceptable only when a cell was sectioned precisely meridionally, as determined by the simultaneity with which daughter nuclear structures appeared; (*b*) measurements were discarded if it proved impossible to obtain two values of W_{cr} (both sides of the furrow) simultaneously with D_{min} from nearly adjacent sections; (*c*) all measurements from a given cell were discarded if S_n deviated by more than 10 μ from the mean curve in Fig. 6 *b*. 32 cells conformed to all of these standards. Numerous cells were disqualified because of failure to meet criterion (*a*). 12 cells were eliminated because of vagaries of electron microscopy interfering with fulfillment of criterion (*b*). Two cells were judged aberrant according to criterion (*c*).

Dr. S. B. Carter generously supplied the cytochalasin B. It was dissolved in dimethyl sulfoxide, according to Carter's (1967) recommendation, at 0.1% concentration. This stock solution was stored at −10°C. Small volumes of the stock solution were diluted in calcium-free sea water before use. Dissolution was facilitated by gentle agitation.

RESULTS

Over-All Features of the Contractile Ring

Cytokinesis proceeds equatorially and approximately symmetrically in *Arbacia* eggs, as in most cells. Before this event, that is, from the time of ovulation to the end of mitotic anaphase, each egg is perfectly spherical, with a diameter of about 75 μ. Representative stages in this sequence are illustrated in Figs. 1 *a*, 2 *a*, 3 *a*, and 7. As cleavage commences, an egg momentarily appears cylindrical or barrel-shaped before assuming the characteristic dumbbell shape as the cleavage furrow progressively constricts at the equator. Demembranated *Arbacia* eggs grown in calcium-free sea water at 20°C complete the first cleavage furrow in about 6 min (Scott and Pollen, 1951). Such eggs develop normally except that calcium-free sea water prevents proper formation of the hyaline layer which reduces adhesion between daughter blastomeres. At the end of cleavage, daughter blastomeres remain attached only by a narrow connection (the midbody), which lingers for an undetermined length of time.

The contractile ring (CR) of *Arbacia* is discernible in electron micrographs as a curved band of closely spaced microfilaments. The band smoothly fits and undoubtedly forms the contours of the cleavage furrow. In meridional sections both the furrow and the CR are concave (Fig. 2 *b*) whereas in equatorial sections the CR is of course convex (Figs. 4 *b–c*). When reconstructed in three dimensions, the CR resembles a spool—that is, a figure generated by rotating a U about a horizontal axis (that of the cell).

The CR is a compact, seemingly cohesive organelle. It occupies cytoplasmic space just beneath the plasma membrane of the cleavage furrow. No recognizable structures have been observed between this membrane and the CR. Its presence creates a "zone of exclusion," in the sense that other recognizable cytoplasmic structures are virtually always restricted from the realm of the CR. Excluded elements include yolk granules, mitochondria, the large echinochrome granules (whose contents are routinely extracted in preparations illustrated here), stem-bodies, endoplasmic reticulum, unidentified vesicles, and smaller granules such as ribosomes and glycogen. The CR is only rarely discontinuous; occasionally a single echinochrome granule is inserted into the CR and is found very near the plasma membrane. Portions of the cell surface not actually underlain by the contractile ring exhibit most of the aforementioned structures pressed directly against the plasma membrane (Figs. 1 *b* and 3 *b*).

The CR is smoothly curved, conforming closely with the *over-all* curvature of the egg. In contrast, the plasma membrane proper is highly irregular in its contour (Fig. 2 *b*). Numerous microvillus-like projections (measuring about 0.5 by 6 μ) decorate the entire surface of every egg, in furrows and at poles alike. Unlike true microvilli in many tissues, these processes are virtually devoid of microfilaments in their cores As a consequence of these many processes, the CR actually contacts the plasma membrane at only some points. Contact with the membrane is not made beneath microvillus-like projections. Another remarkable aspect of the CR's curvature is that its width corresponds very precisely with the width of the *concave* profile of the cleavage furrow, in the vast majority of cases (see Fig. 2 *b*). The edges of the CR correspond to the inflection points where concavity turns to convexity, even when the CR is somewhat asymmetrical—one "limb" measuring more than the other. These observations provide compelling evidence for a distinct causal relation between the presence of the CR and cell constriction.

Microfilaments of the Contractile Ring

Microfilaments of the CR in *Arbacia* are aligned circumferentially; they appear as transversely sectioned profiles in meridional sections (Figs. 2 *b* and 5), and as longitudinal filaments in equatorial sections (Figs. 4 *b–c*). Individual microfilaments are difficult to identify as discrete structures under either condition (Figs. 4 *c* and 5). Difficulties in discerning individual filaments probably reflects their small diameter, their close packing arrangement, and the possibility that they are associated with some nonfilamentous, dense material. In thin equatorial sections which afford sufficient separation between filaments, CR filaments measure 35–60 A in diameter. So far as this investigation has shown, CR filaments are all the same size—no thicker fibers of 80 A or more have been observed anywhere in the cell. CR filaments usually appear slightly wavy. Individual filaments have not been traced with complete certainty beyond 0.25 μ in length.

In Fig. 5, individual microfilaments in cross-sectional view are identified where they are particularly prominent. Their range of diameters is 45–60 A. Each microfilament profile appears to consist of a dense circle with a less dense center, as if the filament might be "tubular" in some sense. (True microtubules are nearly an order of magnitude larger.) In a few cases, a filament profile is surrounded by a loose cloud of amorphous material extending the total diameter to about 175 A (triple arrow, Fig. 5).

Other elements of the CR exclusion zone present an ambiguous morphology; strands of dense material (arrows, Fig. 5) may also be filamentous in composition but merely unidentifiable as such because of unfortunate orientation. Alternatively, these masses may represent additional material among and between the microfilaments of the CR.

The present investigation revealed no evidence that filaments are branched. No changes in lateral spacing of CR filaments during cleavage were noted, although this determination would be difficult to quantitate. Contrary to Tucker's (1971) recent evidence for bridges between CR filaments, none were found in *Arbacia*. Likewise, specialized associations between CR filaments and other organelles, in particular the plasma membrane, were not clearly discerned; a few submembrane dense patches were discovered (Fig. 4 *c*), but their composition and possible significance are unknown.

When Does the Contractile Ring First Appear?

In Figs. 6 *a–b*, morphometric determinations at different stages lend a sense of the dynamics of the CR during the cleavage sequence. According to this study, every cell with a discernible cleavage furrow—even if very shallow—possesses a seemingly well-formed contractile ring (see Fig. 6 *a*). At its first appearance, the CR is about 5 μ wide and 0.1–0.2 μ in thickness.

Conversely, all precleavage eggs (i.e., ones which are still spherical) exhibit no indications of a preexisting CR or display any recognizable signs

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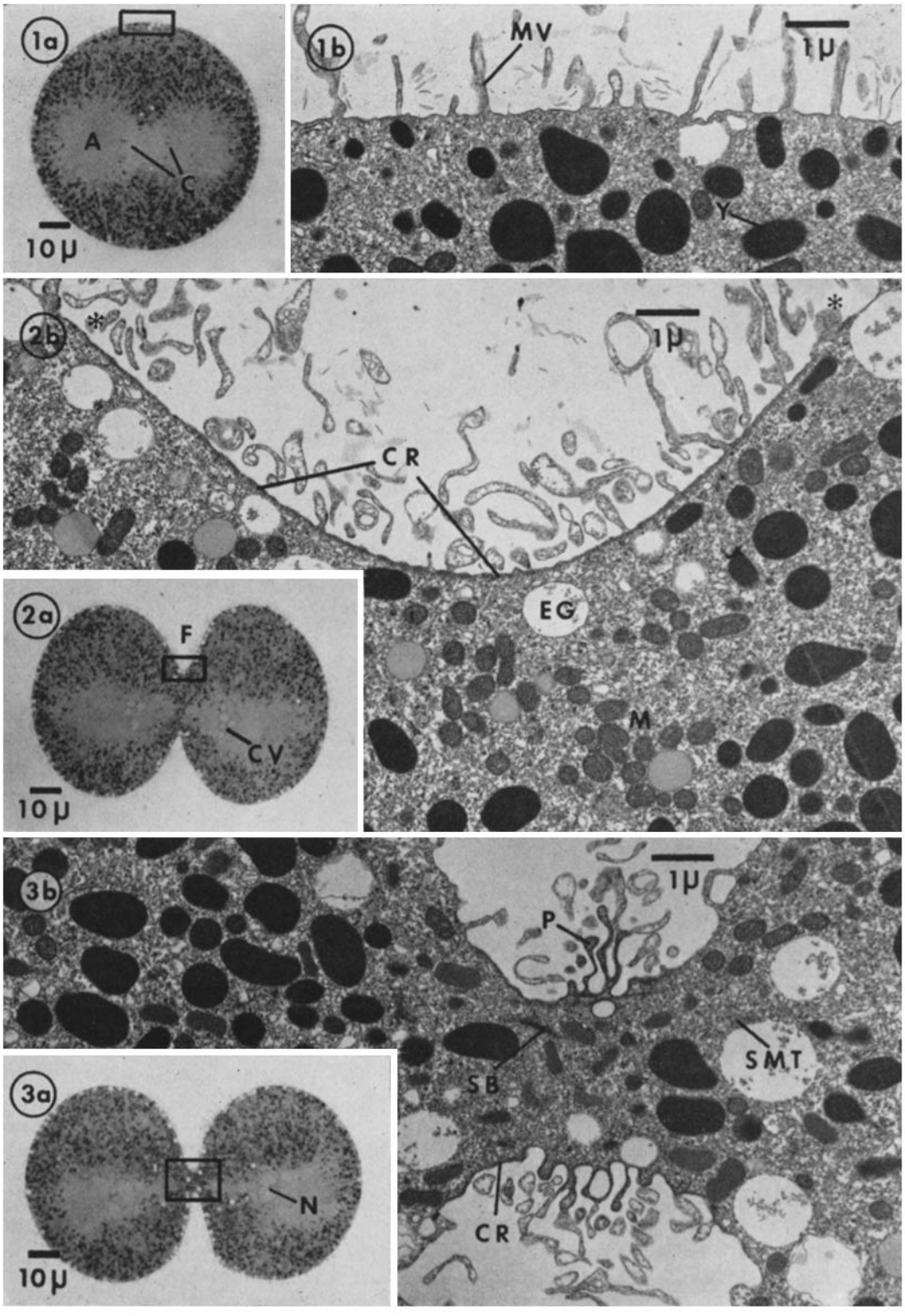
FIGURE 1 (*a*) Meridional section of an *Arbacia* egg at late anaphase, about 30 sec before furrowing begins. Daughter sets of chromosomes (*C*) are nearly maximally separated but the egg is still spherical. *A*, yolk-free zone of mitotic aster. The box frames the field of Fig. 1 *b*. ×490. (*b*) Presumptive furrow region of the same cell shown in Fig. 1 *a*. Neither cleavage furrow nor contractile ring is yet present. *MV*, microvillus-like projection; *Y*, yolk granule. ×9500.

FIGURE 2 (*a*) Meridional section about 4 min after cleavage has begun. Clusters of chromosomal vesicles (*CV*) characterize the reforming nuclei at this stage. *F*, cleavage furrow. Box corresponds to Fig. 2 *b*. ×490. (*b*) The contractile ring (*CR*) is readily seen in this half-cleaved egg (not the same specimen as Fig. 2 *a*). The *CR* spans the concave surface (between asterisks). The *CR* prevents cytoplasmic particles, yolk, mitochondria (*M*), and echinochrome granules (*EG*) from coming into direct contact with the plasma membrane. ×9500.

FIGURE 3 (*a*) Meridional section at late cleavage, about 5 min after its onset. Daughter nuclei (*N*) are now completely reformed. Box corresponds to Fig. 3 *b*. ×490. (*b*) The contractile ring (*CR*) is waning and is about to disappear in this egg about 5.5 min after it began to cleave. Complex protrusions (*P*) occur at this stage; the plasma membrane associated with them appears particularly dense. Stem-bodies (*SB*) and a few spindle microtubules (*SMT*) are remnants of the mitotic apparatus. ×9500.


1a
A
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10 μ
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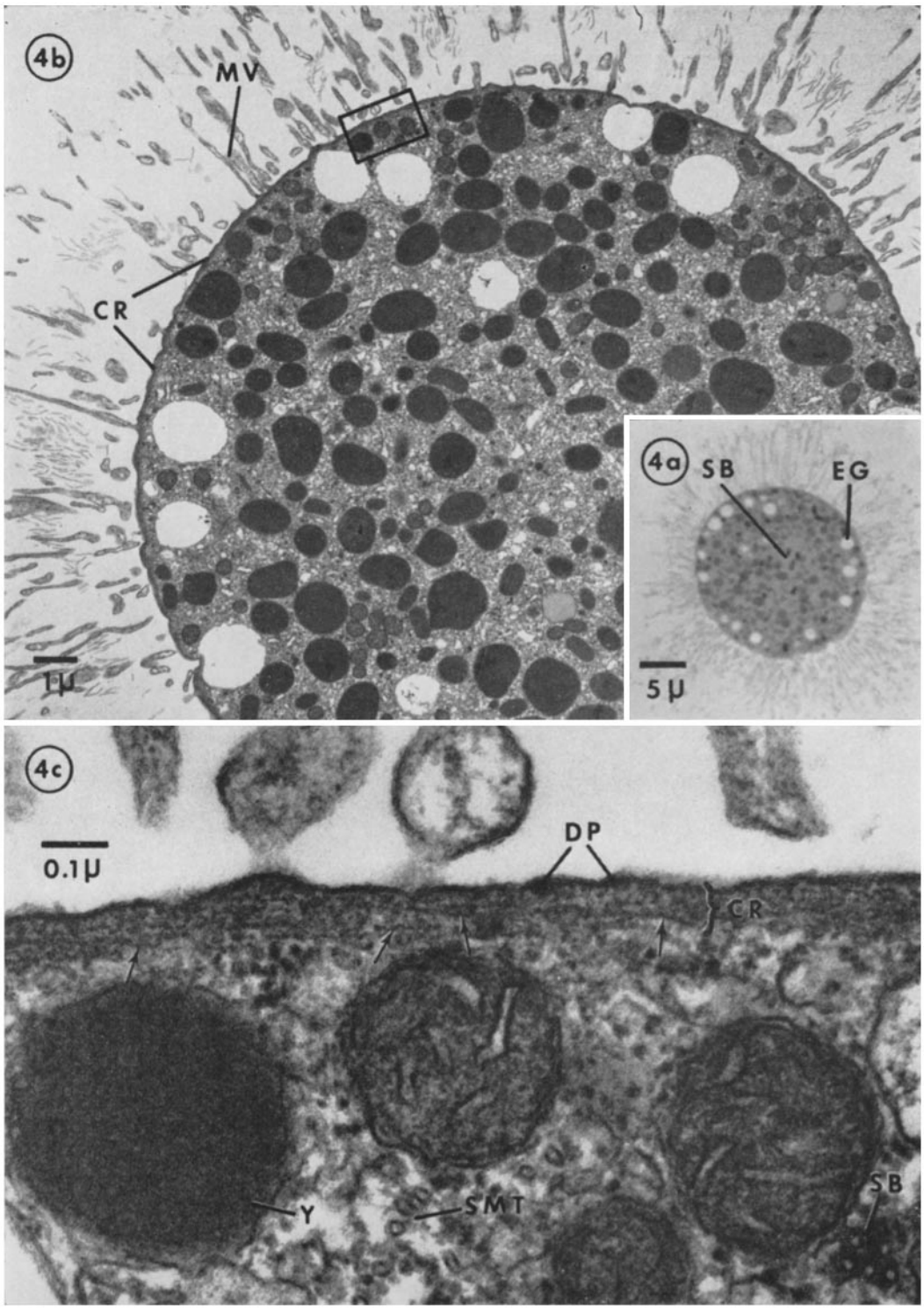

FIGURE 4 (*a*) Light micrograph of a precisely equatorial section from a midcleavage cell similar to the one in Fig. 2 a. Echinochrome granules (*EG*) and stem-bodies (*SB*) stand out as the lightest and darkest bodies. ×1500. (*b*) Electron micrograph from a section adjacent to Fig. 4 *a*. The contractile ring (*CR*) completely encircles the egg without interruption. Microvillus-like projections (*MV*) radiate for several microns. Box frames Fig. 4 *c*. ×6500. (*c*) Higher magnification displays organization of the contractile ring (*CR*) and individual *CR* filaments (arrows). The entire band of *CR* filaments is closely associated with the plasma membrane, but no specialized adhesion sites are discernible, unless they are the occasional dense patches (*DP*). *Y*, yolk granules; *SB*, stem-body seen in cross section; *SMT*, spindle microtubules. ×100,000.

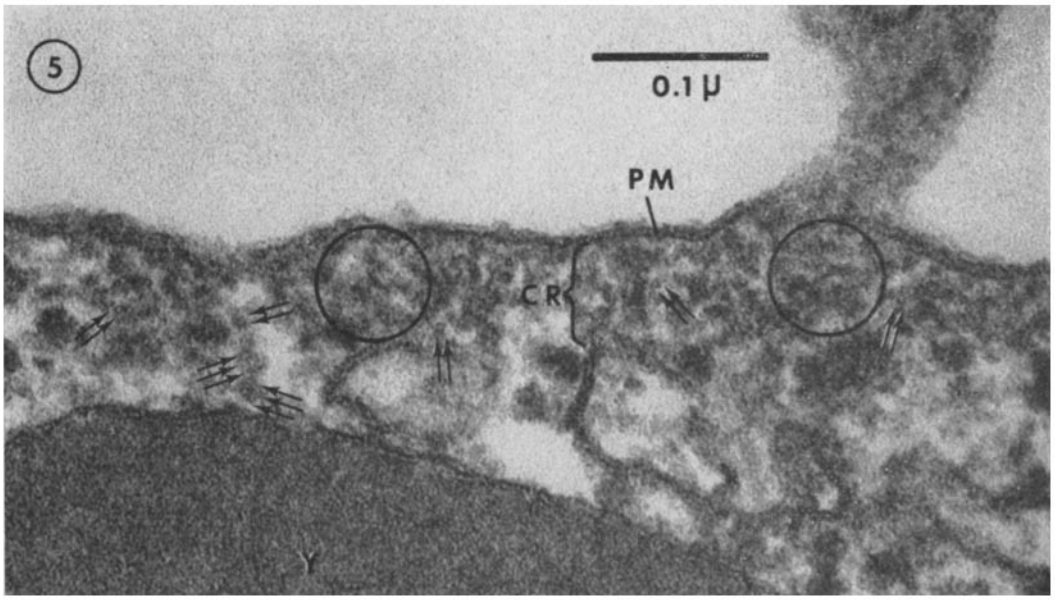

FIGURE 5 In a meridional section (corresponding to an enlargement of Fig. 2 *b*) the contractile ring (*CR*) appears to be constituted of poorly defined meshes of dense material in short strands about 40–80 A thick (encircled) and discrete circular profiles (double arrows) which are thought to represent individual *CR* filaments. They appear to be in some sense hollow or tubular. Some of these profiles (triple arrows) are surrounded by aggregations of less dense material. *PM*, plasma membrane; *Y*, yolk granule. Instrumental magnification: ×80,000. Final magnification: ×228,000.

of a CR-assembly process. At least one egg was examined which was at the extreme end of anaphase (S_n = 20 μ) and still spherical (D_{min} = 72 μ). It failed to show the slightest morphological indication of a CR when examined ultrastructurally. Its equatorial cortical cytoplasm and surface features were indistinguishable from the cell in Fig. 1 *b*, which is also in anaphase.

Two other specimens displayed very early signs of cleavage (equatorial diameter reduced only to 66 μ); the contractile ring was found to be present and quite prominent in each cell. Time-lapse cinemicrography of dividing *Arbacia* eggs has established that this degree of cleavage is achieved less than 20 sec after the end of anaphase chromosome movements (which themselves last about 1 min). Therefore, the accuracy of the present study permits the conclusion that the contractile ring is assembled within 20 sec of the end of anaphase. Moreover, cleavage begins virtually simultaneously as the CR appears.

When Does the Contractile Ring Vanish?

Very late in cleavage, when the equatorial diameter of a cell is reduced to about 6 μ, the width of the CR abruptly decreases (see Fig. 6 *a*). At a diameter of 2.5 μ, the CR is no longer discernible, and cleavage, as formally defined, is concluded. At this stage a midbody is present, albeit not so compact as midbodies in other cells (Byers and Abramson, 1968). The latest stage to exhibit a CR is at D_{min} = 4.5 μ—slightly later than the cell in Fig. 3 *b*, which also possesses a scanty CR.

The ultimate fate of the contractile ring is presently unknown. However, it may be noteworthy that, in the final phase of cleavage, traces of an unidentified dense material accumulate in association with a unique form of cytoplasmic protrusion (Fig. 3 *b*). Since this material appears just as the CR wanes, one may speculate that the two structures are somehow related. If one accepts that CR microfilaments per se are thoroughly disassembled at the end of cleavage, then perhaps the observed dense material is yet another component of the CR which remains behind. As an hypothesis, this substance could be involved in bonding CR filaments to the plasma membrane; in the absence of CR filaments, the substance might remain adherent to the membrane in a way that alters the

membrane's physical properties sufficiently to explain the observed protrusions.

Does the Volume of the CR Remain Constant?

This important question arises out of the hypothesis that CR filaments generate a motive force by interacting in a sliding fashion analogous to muscle. If filament sliding is the principal process of contraction, the CR filaments should increasingly overlap one another as annular constriction proceeds. In the absence of any change in lateral spacing between filaments, the volume of the CR (V_{cr}) should remain constant throughout the process. To be logically complete, the postulate also assumes that there are no alterations in total filament length, as by terminal addition or subtraction. This postulate (hereafter referred to as the simplified sliding-filament mechanism) may be tested by morphometric determinations.

Resolution of this problem actually depends upon three separate determinations: what are the thickness (T_{cr}), width (W_{cr}), and circumference ($\sim D_{min} \times \pi$) of the CR? The latter is fortunately also an inverse linear function of time, since the cleavage rate is constant (Scott and Pollen, 1951).

The thickness of the CR has been measured in a large number of cells embracing the entire range of cleavage stages. So far as can be determined, this parameter, T_{cr}, remains constant at about 0.1–0.2 μ. T_{cr} shows no meaningful variation as a function of cleavage stage or region of the CR.

In preliminary studies, the width of the contractile ring (W_{cr}) was found to vary from specimen to specimen. The variability bore an ambiguous relationship to the extent of cleavage, so a concerted effort was made to collect enough morphometric data to clarify any possible correlation between W_{cr} and equatorial diameter (D_{min}). (The latter dimension converts easily to the circumference of the CR at its narrowest point). Strict criteria were applied in collecting these data, as outlined in Materials and Methods. Accumulated measurements from 32 acceptable cells are plotted in Fig. 6 *a*. The data still show considerable scatter (range = 2.5–17 μ), but in a general sense some valuable inferences may be drawn from it. There is little consistency to the variability except possibly during the first half of cleavage (D_{min} = 75–40 μ). W_{cr} may show a gradual increase from 5 to 8 μ during this period. For present purposes, however, the data are interpreted to show that W_{cr} displays essentially no change during all of cleavage except for that which is inherent between individual cells; according to this view, W_{cr} measures about 8 μ at all times.

A useful reference curve in Fig. 6 *a* is labeled "$V_{cr} = k$." As defined in Materials and Methods, this theoretical curve predicts values for W_{cr} if the

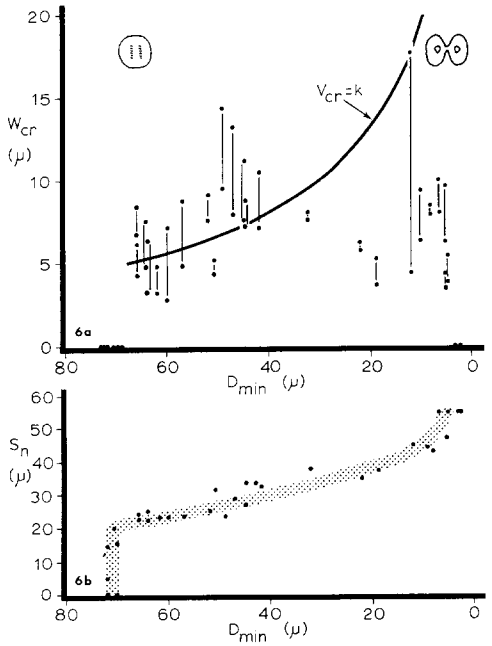

FIGURE 6 (*a*) This graph plots changes in width of contractile rings (W_{cr}) as a function of extent of cleavage—expressed as minimum equatorial diameter (D_{min}). Each pair of data points connected by a vertical line represents measurements from opposite furrow profiles of a single egg. The measurements are taken from electron micrographs of precise meridional sections. Considerable variability in the width (W_{cr}) is displayed between one side of a furrow and another, as well as between eggs. On the whole, W_{cr} does not change enough to support the constant volume hypothesis. Actual values for W_{cr} depart substantially from the theoretical curve, $V_{cr} = k$, indicating that the volume of the CR diminishes as cleavage proceeds. This graph also depicts the abrupt appearance and disappearance of the CR coincident with the onset and conclusion of cleavage. (*b*) This graph plots the increasing distance between daughter nuclei (S_n) as a function of the extent of cleavage (D_{min}). Stippling indicates the averaged curve. The effect is not surprising, but the quality of the curve is useful in lending reliability to the previous graph (Fig. 6 *a*); correlated functional processes appear to be morphologically preserved in these preparations of *Arbacia* eggs. The same cells have furnished the data for both graphs.

volume (V_{cr}) does remain constant, assuming that (*a*) T_{cr} is constant, which it is, and (*b*) the initial value of W_{cr} is 5 μ. Beyond the midpoint of cleavage ($D_{min} > 40$ μ), the curve rises very sharply. Since actual data points for W_{cr} clearly do not show a corresponding rise, an unavoidable conclusion may be drawn: *the volume of the CR does not remain constant*, at least during the second half of cleavage. (In general, the reliability of this conclusion is not weakened by the inability of the observed data in Fig. 6 *a* to distinguish empirical from theoretical events specifically during the first half of cleavage. Since data points in the period D_{min} = 75–40 μ do seem to correspond to the curve $V_{cr} = k$, the possibility of a bimodal mechanism of contraction cannot be excluded. During the first half of cleavage, V_{cr} *may* remain constant—a possibility that would be consistent with the simplified sliding-filament hypothesis).

However, during the second half of cleavage, V_{cr} obviously declines considerably. Thus, the simplified sliding-filament hypothesis cannot apply to the process of cell cleavage. Some implications of this conclusion, and suggestions for altering the hypothesis, will be discussed presently.

Temporal Relations between Cleavage and Nuclear Division

A causal relation between mitosis and cleavage induction has been established by Rappaport (1965) and others. In temporal terms, cleavage induction occurs during a very brief period at the end of anaphase. The process linking mitosis and cleavage induction, however, is not known.

The present study confirms that mitotic anaphase is completed before cleavage begins in *Arbacia* eggs. As shown in Fig. 6 *b*, daughter chromosomes are separated by at least 20 μ before the first indications of a cleavage furrow. Time-lapse films of these events indicate a very brief delay (~20 sec) between the end of anaphase and the inception of the cleavage furrow, as previously mentioned.

The first ultrastructural signs of the contractile ring (and of equatorial constriction) coincide with the earliest observable signs of nuclear envelope restoration. Chromosomes are small, compact, and densely staining in sections throughout all phases of mitosis (see Fig. 1 *a*). They lack investing cisternae of nuclear envelope material. However, at the very onset of cleavage, nuclear envelopes are rapidly reconstituted around individual chromosomes. Each chromosome suddenly thereafter disperses within its enveloping membranes. These lightly staining "chromosomal vesicles" then swell and gradually coalesce. Daughter nuclei are finally restored after a few minutes, when the equatorial diameter is about 12 μ. Some stages in this process are seen in Fig. 2 *a*, 3 *a*, and 7.

Cytoplasmic cleavage is spatially and temporally linked with mitosis. Rappaport (1965, 1971) summarizes evidence which strongly implicates the asters of the mitotic apparatus in a specific induction process. Although its mechanism is unknown, this process stimulates the onset of cleavage. In view of present knowledge of the CR, and recognizing that the mitotic apparatus is largely composed of microtubules, attention has been given to various microtubule populations in this study. Possible morphological interactions between the CR and microtubules have been sought shortly before and during cleavage.

In Figs. 1 *a*, 2 *a*, and 3 *a*, astral zones of the mitotic apparatus appear as yolk-free areas surrounded by trains of cytoplasmic organelles radiating outward from the spindle poles. Inexplicably, however, very few aster microtubules have been observed at any of these stages. Consequently, no evidence has been obtained in this study which either supports or refutes the postulate that mitotic asters (qua microtubules) mediate the induction of cortical contractility.

Most spindle microtubules come to aggregate as stem-bodies at the end of anaphase. Most of these are clustered within a 10 μ radius of the egg's central axis even before cleavage begins. It is only at a moderately late stage of cleavage that any of these microtubular elements approach the general vicinity of the contractile ring. The deepening furrow eventually squeezes this sheaf of microtubules and stem-bodies, which, however, never become embedded in the substance of the CR (Figs. 4 *a–c*). In other words, microtubules are never intimately associated with CR filaments at any stage of cell division.

Effects of Cytochalasin B

Furrowing is abruptly arrested in dividing *Arbacia* eggs when treated with dilute cytochalasin B solutions, regardless of the stage of cleavage. Mitosis is altogether unaffected. When the drug is applied before late anaphase, a cleavage furrow simply fails to appear. Effects during the course of cleavage depend upon the depth of furrowing

achieved at the time of application, as summarized in Fig. 7. During the first third of cleavage, cytochalasin B brings about rapid regression of the furrow constriction until the egg is once again spherical, a process requiring about 3 min. The initial action of the drug on the advancing furrow is observed within 60 sec. When furrowing is arrested beyond the halfway mark of cleavage, some regression is typically observed but the furrow does not entirely disappear. For technical reasons associated with orientation of the specimen, eggs in this condition are easier to study microscopically than ones which are spherical.

Regardless of the time of treatment or the dosage, nuclear division continues unabated and at a seemingly normal rate in eggs treated with cytochalasin B. Prometaphase and anaphase chromosome motions are unaffected by the drug, as are reconstitution of nuclear envelopes, chromosome dispersion in the vesicles and fusion of chromosomal vesicles. Microtubules of the mitotic apparatus suffer virtually no morphological alterations; the single exception is that a small number of spindle microtubules are slightly dislodged or disoriented. Some microtubules come to lie perpendicular to the axis of mitosis. This displacement presumably arises as a secondary effect of furrow regression and the accompanying shifts of cytoplasm.

Various concentrations of cytochalasin B were applied to eggs shortly before the expected onset of cleavage in order to determine the approximate threshold concentration for inhibition of cleavage. Concentrations of 0.5–10 μg/ml arrested furrowing in the manner described above. 0.1 μg/ml elicited no observable effect. Concentrations of 0.2–0.4 μg/ml yielded graded effects in which proportions of affected cells varied with the concentration. The threshold dosage of 0.5 μg/ml equals 1.04×10^{-6} M. The viability of all treated cells was verified by observing successive mitotic divisions. Control eggs were treated with dimethyl sulfoxide alone, the intermediary solvent for dissolving cytochalasin B (Carter, 1967). Eggs were unaffected by concentrations up to 1%, the amount present in 10 μg/ml solutions of cytochalasin B.

The microscopic morphology of drug-treated cells is distinctive in at least three ways: first, cytochalasin B inhibits furrowing activity; second, the contractile ring is entirely absent; and third, the drug causes a relaxation of the surface rigidity which ordinarily maintains the spherical shape of the egg. This latter effect is manifest as deformities, surface irregularities, and occasional blebbing, seen in Fig. 8 *a*.

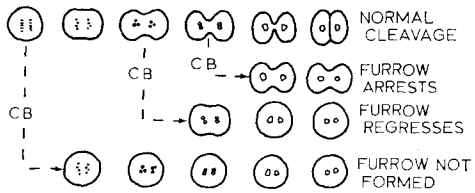

FIGURE 7 Diagram depicting various effects of cytochalasin B (*CB*) applied at different times in the cleavage sequence. Nuclear division is never altered. The cleavage furrow either regresses completely or arrests depending on the time of application of the drug (early or late, respectively).

In a cell where the furrow has been partially relaxed but is still evident after exposure to cytochalasin B, the contractile ring may be sought at the base of the furrow, as in Fig. 8 *b*. The band of contractile ring filaments is simply not in evidence and has been replaced by an expanse of undifferentiated cytoplasm. For the most part, cytoplasmic particles and large echinochrome granules extend right up to the plasma membrane of the previously active furrow. Therefore, cytochalasin B evidently causes the rapid and thorough degradation of contractile ring filaments, and other structures swiftly migrate outward to occupy the vacated space.

The cell surface is highly irregular after cytochalasin B treatment. The number of microvillus-like projections appears less. At certain points along the plasma membrane of the furrow there are small localized patches of opaque material (arrows, Fig. 8 *b*). The material in these dense patches does not display a microfilamentous substructure or any other ultrastructural characteristics of the normal contractile ring. Scanty as this material is, it resembles the dense submembrane substance which appears at the end of cytokinesis (Fig. 3 *b*), as previously mentioned. Both of these substances may derive from the disassembly of the contractile ring.

DISCUSSION

Dynamic Assembly and Disassembly of Contractile Ring Filaments

This investigation demonstrates that the contractile ring in *Arbacia* is a highly transitory organelle. Its initial ultrastructural manifestation occurs about 20 sec after completion of anaphase chromosome motion as an equatorial band of

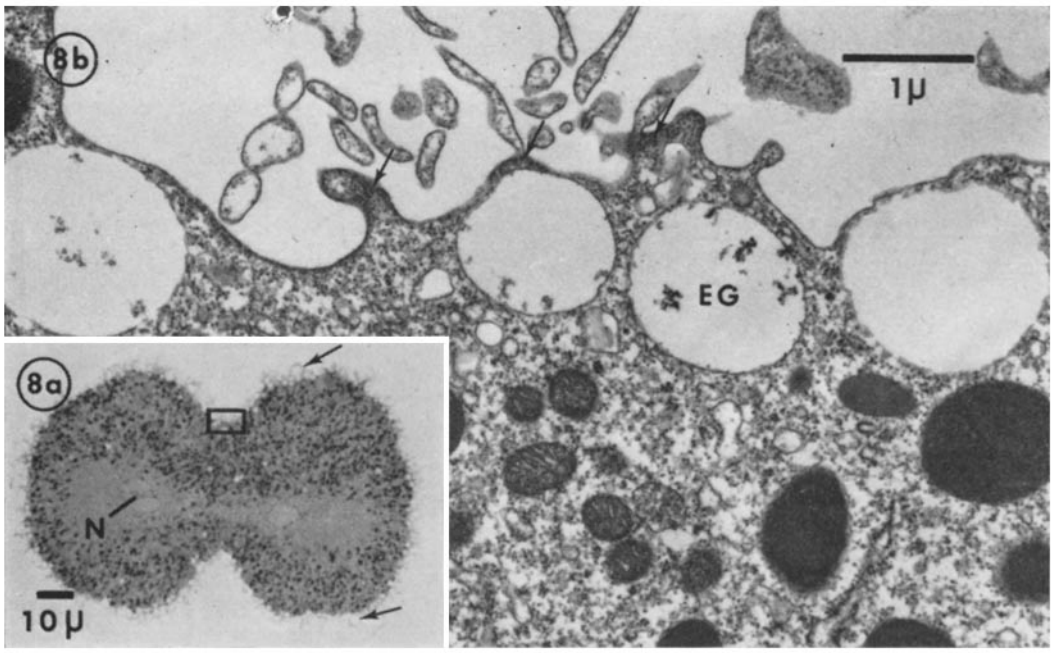

FIGURE 8 (*a*) *Arbacia* egg treated with cytochalasin B (1 μg/ml) for 1 min before it was fixed. Compared with Fig. 3 *a*, the nuclear morphology (*N*) of this egg indicates that it has reached a late cleavage stage. However, the depth of the furrow is more typical of an earlier stage, indicating considerable furrow regression. Irregularities in the egg's surface suggest that a general relaxation of cortical tension has occurred. Arrows point out spherical protrusions which are surface blebs. Box frames Fig. 8 *b*. ×490. (*b*) The contractile ring cannot be identified here, after only 1 min exposure to cytochalasin B. Vague patches of density (arrows) perhaps represent remains of the degraded contractile ring. *EG*, echinochrome granule. ×17,200.

microfilaments 4–8 μ wide and 0.1–0.2 μ thick. Throughout its brief existence of 6–7 min, the CR remains intimately associated with the cleavage constriction, both temporally and spatially. Neither its width nor thickness changes appreciably during this time (see Fig. 6 *a*), although its circumference decreases. The CR suddenly disappears during the final 30 sec of cell constriction, leaving behind some residual dense material beneath the plasma membrane of the midbody. This very brief existence and rapidity of CR-assembly and disassembly attest to the formidable dynamic nature of this organelle. These unique features of the contractile ring distinguish it from other known contractile machinery, which tend to be more stable.

Origin of the Contractile Ring

The nature of the assembly process is open to speculation, as is the ultimate source of CR filaments. No precursors of CR filaments have yet been observed. A rapid linkage of globular subunits into filaments is one conceivable mechanism of assembly. Szollosi's (1970) hypothesis that CR filaments are derived from intact core filaments of microvilli is not supported by present findings; in *Arbacia*, microvilli possess few core filaments and the microvilli are not depleted at the site of the cleavage furrow.

Several facts point to an involvement of the plasma membrane in CR-assembly. Being composed primarily of circumferentially aligned microfilaments, the CR is a cohesive zone-of-exclusion by which cytoplasmic organelles, particles and granules are almost totally separated from the plasma membrane. This observation argues that the CR is actually assembled *in situ*, and rapidly, beneath the plasma membrane. Consequently, one can conceive of the plasma membrane as a template or forming-surface against which the CR is assembled, as depicted in Fig. 9. Accordingly, CR-

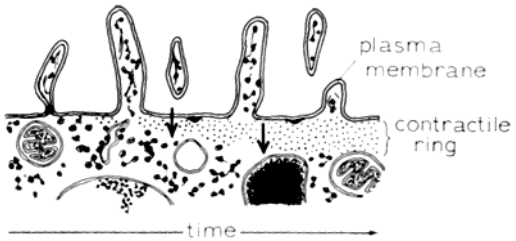

FIGURE 9 Schematic representation of an hypothetical assembly process for the contractile ring, showing progressive deposition of contractile ring filaments (here shown as dots, as if in meridional section) on the underside of the plasma membrane. This rapid accumulation forces all cytoplasmic organelles and inclusions inward away from the plasma membrane, in the direction of the arrows. Assembly of the contractile ring probably occurs in less than a minute.

assembly may be visualized as a process whereby microfilaments condense or polymerize in direct contact with the plasma membrane and build up to the full thickness of the CR while inwardly displacing cytoplasmic organelles and inclusions. The forces which might trigger or direct these events are unknown, but it may be fruitful to think of a collaboration between mitotic apparatus and plasma membrane in this effort. This scheme, shown in Fig. 9, is consistent with all described contractile rings, except for the protozoan *Nassula* (Tucker, 1971) in which a girdle of microtubules is elaborated between the CR and plasma membrane. In all other forms the CR is directly beneath the plasma membrane.

Role of Contractile Ring in Cleavage

There are several kinds of observations which comprise the strong circumstantial evidence that contractility of the CR causes cleavage. Among these is the very close temporal and spatial coincidence between the CR and the cleavage process in dividing *Arbacia* eggs, as shown here. The microfilamentous ultrastructure of the CR is further indication of its contractile nature—by virtue of analogy to other well-documented reports of microfilament-dependent contractility in a variety of systems (Cloney, 1966, 1969; Cloney et al., 1971; Gingell, 1970; Luckenbill, 1971; Schroeder, 1970 *a*, 1971; Wessells et al., 1971). Moreover, cytochalasin-arrested furrowing activity is accompanied by the selective loss of CR filaments, which substantially demonstrates the dependence of cleavage function on the CR as an intact ultrastructural entity.

But how does the CR contract? An objective of this investigation was to test the validity of a simplified sliding-filament mechanism (see hypothesis set forth in Introduction). Had the results shown that the volume of a CR remains unchanged during cleavage (i.e. that $V_{cr} = k$, see Fig. 6 *a*), then a mechanism of CR contraction based upon filament sliding might have been confidently advanced. However, the evidence in Fig. 6 *a* establishes rather emphatically that the volume of the CR actually *decreases* during cleavage.

The strong conceptual appeal of a sliding-filament mechanism recommends a reevaluation rather than outright rejection of the hypothesis as it applies to cell cleavage. The results perhaps do more to underscore the dynamic nature of the CR than to rule out strictly a sliding-filament mechanism of action. Clearly, the so-called "simplified" sliding-filament version advanced here is inadequate to explain the observations; in the case of *Arbacia* eggs, it appears that a static array of sliding filaments does not accurately portray events in the CR during cleavage. After its assembly, progressive CR-disassembly may very well mask any expected dimensional changes in the CR, which could otherwise be attributed to filament sliding in a simple sense. Therefore, the chief limitation of the hypothesis which this investigation set out to examine may be the implication that the contractile ring is composed of a static population of microfilaments. In actuality, a sliding interaction between CR filaments may still represent the force-generating event, but rapid disassembly also occurs, indeed perhaps as a consequence of contraction.

The data in Fig. 6 *a* are consistent with a mechanism whereby the CR is "consumed" by its own contractility. To the degree that this notion is a useful one, the process of consumption or disassembly appears to occur uniformly throughout the CR since both its width and thickness remain essentially constant. Further assessment of the sliding-filament hypothesis may require careful determination of some of the following aspects: average or unit length of CR filaments; identification of polarity in CR filaments; extent of "active" overlap (if any) between interacting filaments; more about the nature of filament interaction; and localization of the disassembly process relative to individual filaments.

Nature of Cytochalasin Effects

Thanks to Carter's (1967) pioneering work with cytochalasin B in which he describes its most apparent biological effects, we have known that the drug somehow inhibits cleavage without preventing mitosis, at least in cultured cells. An electron microscope study of cultured HeLa cells (Schroeder, 1970 *c*), as well as a preliminary report of the present study (Schroeder, 1969), revealed that the contractile ring is rendered indiscernible by cytochalasin treatment and that cleavage activity is quickly abolished, presumably as a direct result. The present findings amplify these observations by demonstrating that cleavage is actually arrested in midcourse. Furthermore, they lead to the conclusion that the drug primarily affects microfilaments rather than cell-to-substrate adhesion, as originally proposed by Carter (1967), or membrane fusion between sibling cells, as suggested by Estensen (1971).

Arbacia eggs have provided an opportunity to observe the immediate effects of cytochalasin B. As previously reported (Schroeder, 1969), furrowing activity is discernibly arrested within 60 sec of treatment. Recent time-lapse studies of this process (Schroeder and Cloney, unpublished) confirm that furrow advance ceases within 20 sec and that the furrow begins to recede within 40 sec. Cytochalasin-arrested cells rapidly lose their contractile rings. In *Arbacia*, this has been found within 1 min after treatment. The possibility that the CR is merely dislodged, rather than actually degraded by the drug, has been ruled out.

Previous studies describe the appearance of felt-like aggregations or finely granular masses in cells after cytochalasin treatment (Godman et al., 1971; Lash et al., 1970; Schroeder, 1970 *c*; Wessells et al., 1971); these are thought to signify microfilamentous material in a state of degradation. Similar masses were not found in *Arbacia* eggs. The only recognizable remnants of the degraded organelle are patches of dense material intimately associated with the plasma membrane. Since similar patches occur in untreated cells at the midbody stage, it is conceivable that these structures are related. Each kind of patch may signify the presence of a remnant substance after the microfilaments are disassembled or degraded. One candidate for this substance— otherwise not accounted for—is the hypothetical material responsible for adhesion between CR filaments and the inner aspect of the plasma membrane. The logical need for such a substance has been considered in the previous article of this series (Schroeder, 1970 *c*). The substance may correspond structurally and functionally to submembrane dense material in the "transverse segments" of intercalated discs between cardiac muscle cells (Fawcett and McNutt, 1969).

The short-term effects of cytochalasin B on HeLa cells and *Arbacia* eggs appear to be straightforward: rapid cessation of furrowing accompanies loss of contractile ring filaments (Schroeder, 1969, 1970 *c*, this report). However, these effects are not universally observed. It has been reported that in certain mammalian cells in culture, furrowing is not immediately inhibited by cytochalasin B (Carter, 1967; Krishan and Ray-Chaudhuri, 1969; Krishan, 1971). This discrepancy is not presently understood; however, the possibility that the drug exhibits variable potency in different culture media has not been investigated. In amphibian eggs, matters are further complicated; contractile ring filaments remain intact after cytochalasin treatment (Bluemink, 1971), but furrowing activity is abolished nonetheless (Bluemink, 1971; Hammer et al., 1971). In attempting to understand these perplexing observations, it should be recalled that CR filaments in amphibian eggs are anomalously coarser than CR filaments elsewhere; this fact may reflect an increased resistance to structural degradation by cytochalasin even though CR contractility is effectively inhibited.

Amphibian eggs may also be anomalous with regard to requirements for membrane "growth" or "membrane fusion" during cleavage, processes espoused by Bluemink (1971) and Hammer et al. (1971) as possible target functions of cytochalasin. However, these properties are not universal requirements of dividing cells. In the case of *Arbacia* eggs, a mechanical necessity for membrane growth is eliminated by the presence of excess surface membrane residing in numerous microvillus-like projections; moreover, Scott (1960) ruled out this mechanism of division by directly observing the surface behavior of dividing eggs. With regard to membrane fusion, interblastomeric contact (and therefore membrane fusion) was prevented in the present studies by growing cells in calcium-free sea water.

For the moment, then, the contractile ring appears to be the most likely candidate as the causal agent of furrowing during cell cleavage and as the

TABLE I
Comparative Aspects of Contractile Rings

Species of cell	Total diameter	Width of CR	Thickness of CR	Rate of contraction	Reference
	μ	μ	μ	μ/min	
Newt egg	2000	16	0.1–0.2	—	Selman and Perry (1970)
Squid egg	1000	10–20	0.1–0.2	—	Arnold (1969)
Nassula	120	5	0.1–0.2	6*	Tucker (1971)
Arbacia	75	3–17	0.1–0.2	40	this report
Jellyfish egg	60	6	0.1–0.2	—	Schroeder (1968)
HeLa cell	20	10	0.1–0.2	8‡	Schroeder (1970 *b*, *c*)

Dimensions of various CRs are remarkably similar but their rates of contraction, where known, vary considerably.
* This is an average value taken from Tucker's (1971) Fig. 1.
‡ Based upon unpublished time-lapse ciné observations that HeLa cell cleavage takes 8 min at 37°C.

principal target of cytochalasin B in its inhibitory role.

Comparative Biology of Contractile Rings

A bibliography of ultrastructural reports of contractile rings in different cells has been included in a previous paper (Schroeder, 1970 *c*). Since then, a few more may be added to the list (Bluemink, 1970; Hinds and Ruffett, 1971; Selman and Perry, 1970; Scott and Daniel, 1970; Tucker, 1971). There are many fine structural similarities among all of these contractile rings. But a few exceptions are worth pointing out. One exceptional feature of *Nassula* (Tucker, 1971) has already been alluded to—it is the unique case in which an organized structure appears between the CR and plasma membrane. Another curiosity of *Nassula* is the fact that the thickness of its CR increases from 0.2 to 0.4 μ rather than remaining constant as in all other forms. However, this occurrence is perhaps anomalous in other ways, especially since it takes place during an uncommonly slow phase of cleavage at the very end of normal cell division. These features are not shared by any other cell type studied to date. A third and final obvious discrepancy between contractile rings of various species is the consistently greater dimensions ascribed to CR filaments in amphibian eggs (Selman and Perry, 1970; Bluemink, 1971; Schroeder, unpublished), as opposed to all other cell types. Microfilaments of amphibian CRs measure 80–100 A as against 35–70 A in other forms. No explanation of this difference is presently available.

Contractile rings from a variety of species and cell types are remarkably similar in vital dimensions. Table I summarizes available measurements from the literature. Despite variations in cell volume which exceed six orders of magnitude (i.e., newt eggs vs. mammalian cells), the thickness of all CRs consistently measure 0.1–0.2 μ. Their widths exhibit a narrow range of variation (3–20 μ) which is hardly significant relative to the large range in cell size. Although the possible significance of these similarities remains a mystery, it seems unlikely that they are purely coincidental.

Table I also illustrates the rates of contractions—computed as circumferential contraction (i.e., diameter of cell when spherical ÷ duration of cleavage)—vary unpredictably, despite similarities in dimensions of the organelle. Contractile rings in *Arbacia* evidently contract much faster than those of *Nassula* or HeLa cells, though due to no obvious morphological difference. As the rates do not correlate with known cell parameters such as size or physiological temperature, they must reflect as yet undetected metabolic variables governing the contractile capacity of the contractile ring.

Dr. S. B. Carter, Imperial Chemicals Industry, who has been so generous with the results of his experience with cytochalasin B, furnished the sample employed in this study. I acknowledge with gratitude the competent technical assistance of Ms. Constance Comstock and my wife, Eleanor. Thanks are also due Dr. Breck Byers, University of Washington, for critically reading an early draft of this work.

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