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Microfilaments in Cellular and Developmental Processes

Contractile microfilament machinery of many cell types is reversibly inhibited by cytochalasin B.

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Contractility is one of the most fundamental characteristics of cells from higher organisms. Although a great deal is known about contraction in the highly specialized contractile cells of striated muscle, relatively little is known about more primitive contractile systems (*1*). Modern electron microscopy has revealed fine filamentous structures in a variety of animal and plant cell types, and suggestions have been made that the bundles or meshworks of such filaments represent contractile machinery. The drug cytochalasin B now provides a tool to investigate these filaments. As we show in this article, this drug demonstrates a clear correlation between integrity of microfilament systems and various biological phenomena.

Cytochalasin B is known to cause several types of cells to become multinucleate (*2*). The mechanism by which this occurs was discovered by Schroeder (*3*) when he applied cytochalasin to cleaving marine eggs. When the drug was administered to eggs with shallow cleavage furrows, the furrows disappeared and cytokinesis stopped. The spindle continued to function, however, thus giving rise to two nuclei in a single cell. Schroeder found that the drug caused disappearance of the "contractile ring," a band of microfilaments located just beneath the cleavage furrow that is thought to be the contractile agent responsible for pinching the cell in two (*4*).

This incisive experiment has led us to apply cytochalasin to a variety of other cell types that either (i) are known to contain filaments resembling those of the contractile ring in size and structure, or (ii) are, on other grounds, thought to be engaging in contractile activity.

In Table 1 are listed biological processes known to be sensitive to the drug. As will be outlined later, in every case so far examined microfilament morphology is altered by cytochalasin, and an identifiable biological process is concomitantly inhibited. Furthermore, when the drug is removed, filaments reappear and the previously inhibited activity is resumed.

Phenomena that are not affected by cytochalasin are listed in Table 2. In each of these cases in which electron microscopy of cells participating in such processes has been carried out, organelles other than microfilaments have been implicated; for example, microtubules are known to be required for spindle or sperm tail function. Furthermore, though studies are still incomplete, few effects of cytochalasin upon metabolic functions have been found. The general supposition is, then, that cytochalasin acts rather specifically in disrupting the function of contractile microfilament systems of cells.

Microfilaments in Developmental Processes

The main impetus for the study of microfilaments as agents of morphogenesis came from the work of Cloney (*5*) who showed that the remarkably rapid (about 6 minutes in some species) shortening of ascidian tadpole tails during metamorphosis was apparently caused by a microfilament system in epidermal or notochordal cells. Baker and Schroeder (*6*) found that similar filaments appeared in cells of the amphibian embryonic medullary plate and, by their presumed contraction at the outer apical ends of the cells, caused the flat plate to sink downward to form a groove—the precursor of the hollow canal within the vertebrate central nervous system.

The model proposed by Baker and Schroeder, in which the microfilaments act like a "purse string," appears to be a general one for developing epithelial cells in vertebrate embryos (Fig. 1). Thus, invagination of the flat lens placode to form the lens cup, initial elevation of the pancreatic diverticulum, and gastrulation in some vertebrates are all events in which a ring of filaments is found at one end of the participating cells, and at that end narrowing takes place (*7*).

Until Schroeder's elucidation (*3*) of the cytochalasin effect, no tool was available to probe the simple correlation between presence of microfilaments and particular morphogenetic events. We have now explored that relationship in each of the following systems.

Chick Oviduct

The oviduct of a 5-day-old chick is basically tubular and possesses no differentiated cells involved in egg pro-

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Table 1. Processes sensitive to cytochalasin B.

1. Cytokinesis (L fibroblasts, lymphocytes, *Arbacia* eggs, lung epithelium) (*2, 3, 12*)
2. Single cell movement (L and heart fibroblasts, salivary mesoderm, glia) (*2, 13, 21, 28*)
3. Axonal growth cone activity (nerve outgrowth) (*21*)
4. Tubular gland formation in oviduct (*9*)
5. Morphogenesis in salivary epithelium (*13*)
6. Premitotic migration of nuclei in epithelia (oviduct, salivary, lung) (*9, 13*)
7. Cytoplasmic streaming (*Nitella, Avena*)
8. Blood clot retraction (platelets)
9. Tail resorption in tunicate metamorphosis (*Distaplia, Amaroucium, Ciona*) (*36*)
10. Invagination during gastrulation (*Strongylocentrotus, Lytechinus, Dendraster, Urechis*)
11. Smooth muscle contraction (gut peristalsis)
12. Cardiac muscle cell contraction
13. Calcium-induced cortical contraction in eggs (*Xenopus*)
14. Calcium-mediated cortical wound healing in eggs (*Xenopus*)

duction. Administration of estrogen stimulates formation of "tubular glands," whose cells produce ovalbumin, the major protein of egg white (*8*). The initial phase of morphogenesis of a tubular gland is dependent upon microfilaments, as shown by the following results obtained in experiments conducted by Wrenn and Wessells (*9*). Unstimulated oviduct lacks bands of 50-Å microfilaments extending across the inner, luminal end of the cells. From 12 to 18 hours after estrodiol injection, such bands begin to appear. By 36 hours the bands are thicker (Fig. 2A), and groups of cells containing such filaments have already begun to bulge outward into the surrounding mesenchymal stroma. Cells of these primitive glands also contain secretory granules believed to be the storage sites of ovalbumin.

If such a 36-hour oviduct is placed in organ culture in the presence of cytochalasin (*9*), all new gland formation ceases; glands already present regress by sinking back into the oviduct wall, and the microfilament bundles are dispersed. In place of the microfilaments are seen masses of granular and short filamentous material (Fig. 3A). This experiment shows a positive correlation between integrity of the filaments and the morphogenetic process: The filaments are presumed to act as purse strings to narrow the inner ends of groups of cells and thereby cause initial evagination of the tubular glands. Furthermore, it is likely that tension of the microfilament system was actually maintaining the early glandular lumen. This conclusion is based on the observation that, in cytochalasin, such lumina disappear as early glands flatten back into the epithelial wall.

If cytochalasin is removed from the organ culture medium, microfilaments appear once more within the epithelial cells. This occurs even in the presence of cycloheximide ($7 \times 10^{-5} M$), an inhibitor of protein synthesis. Likewise, if estrogen is omitted from the recovery medium, microfilaments reappear although they are fewer in number than when the hormone is present. Thus, the reappearance of microfilaments is not dependent on new protein synthesis or on continued stimulation by exogenous estrogen.

The oviduct case permits two new conclusions. First, estrodiol not only elicits cell differentiation but also causes the organelles responsible for morphogenesis of differentiating cells to be assembled and to function. This is the first case in which a known chemical agent can be shown to cause microfilaments to appear in maturing cells. Second, the integrity of microfilaments seems essential for the early morphogenetic process.

Table 2. Processes insensitive to cytochalasin B.

1. Karyokinesis (spindle function and microtubule integrity) (*2, 3*)
2. Sperm tail function (*Urechis, Ciona, Dendraster*)
3. Ciliary function (*Paramecium, Strongylocentrotus* embryos, *Urechis* embryos) (*2, 46*)
4. Cytoplasmic microtubule integrity (nerve, salivary, oviduct, glia, lung, heart) (*9, 13, 21, 28*)
5. Filament (100 Å) integrity [salivary, oviduct, gila, lung, heart (fibroblast)] (*9, 13, 21, 28*)
6. Neurofilament (100 Å) integrity (nerve) (*21*)
7. Microvillous core filament integrity (oviduct) (*9*)
8. Protein synthesis (salivary, glia, nerve) (*13, 21, 28*)
9. Striated muscle actomyosin superprecipitation (*58*)
10. *Escherichia coli* cell division, flagellar movement, and mating (*59*)
11. Contractile vacuole function (*Paramecium*) (*58*)

Salivary Glands

Morphogenesis of salivary gland epithelium is the classic case of specificity in tissue interactions. Only salivary mesoderm supports epithelial morphogenesis, and in fact the same mesoderm can cause both mammary and bronchial epithelia to branch in a salivary-like pattern (*10, 11*). It is clear that extracellular materials, in part derived from such mesoderm, are essential if epithelial morphogenesis is to occur (*12*). Nevertheless, as was revealed in experiments by Spooner (*13*), it is the system of microfilaments within the epithelial cells that is the key to that phenomenon.

If salivary glands from 13-day mouse embryos are treated with cytochalasin in organ culture, the epithelium flattens into a thin, wafer-like sheet and loses the clefts or branch points formed during earlier morphogenesis (see Fig. 4). Such an epithelium remains quiescent and does not continue morphogenesis while in the presence of the drug (*14*).

The drug produces a striking change within epithelial cells. Control cells contain a ring of microfilaments encircling their inner, luminal ends. In addition, large microfilament bundles extend across the opposite, basal ends of such cells (see Fig. 1). Both kinds of microfilament bundles are absent from cytochalasin-treated cells, and in their place are found large masses of fine filamentous material identical in morphology to those seen in oviduct after drug administration. Cytoplasmic and spindle microtubules and other organelles seem unaffected morphologically by cytochalasin (*15*).

Within 10 hours after cytochalasin has been removed from the culture medium, the salivary epithelium begins to round up and get thicker. At certain points along the edge of the epithelium deep clefts develop (Fig. 4), so that after 18 to 24 hours reversal has occurred and morphogenesis is again under way. Reversed cells once more contain normal microfilament bundles. We have proposed that contractile activity of the luminal ring of filaments (Fig. 1B, *a*) found in all cells is responsible for overall rounding up of the thin

epithelial tissue. Particularly prominent and thick bundles of basal (Fig. 1) filaments are seen in cells at the bases of the newly formed clefts. It is likely that those filaments carry out an accentuated contraction that narrows the basal ends of discrete groups of cells so that they appear to sink inward to form the clefts (*16*).

Both sets of microfilaments reappear in concentrations of cycloheximide ($7 \times 10^{-5} M$) that decrease protein synthesis by 95 percent (*12*). This implies that although disrupted by cytochalasin, the filaments are left in a condition in which they can reassemble without the synthesis of significant amounts of new protein.

The effects of cytochalasin contrast dramatically with those of colchicine, an agent that disrupts several types of microtubules (*17*). Colchicine does halt salivary morphogenesis when applied in organ culture (*12*), presumably because it inhibits mitosis; however, the drug does not cause the original, rounded contours and clefts of the epithelium to be lost. The integrity of microfilaments is not affected by colchicine under conditions in which all cytoplasmic microtubules are disrupted.

If colchicine is applied to salivary epithelia that have first been treated with cytochalasin and have spread into the wafer-like condition, reversal still takes place; the epithelium rounds up, and deep clefts appear once again (*18*). At the concentrations of colchicine used for such experiments, both cytoplasmic and spindle microtubu'es are dispersed and are not visible in treated cells. It seems clear therefore that microtubules do not play a crucial role in reversal; the integrity of microfilaments is sufficient for that process. This result parallels other work on formation of the lens cup (*19*), and the general conclusion can be drawn that microfilaments are probably the active agents in these sorts of morphogenesis. Microtubules are not causal agents, except in chromosome movements and in the maintenance of a columnar cell shape (*20*).

Nerve-Cell Elongation

Elongating axons of embryonic nerve cells are tipped by an expanded "growth cone" from which slender microspikes protrude and wave about. The sole organelle within microspikes and at the periphery of growth cones is a network of fine filamentous material (*21*) (Fig. 2B). Individual filaments of the network are of short lengths and are about 40 to 60 Å in diameter. Neurofilaments 100 Å in diameter and microtubules extend down the axon; most of the tubules terminate at the base of the growth cone, while many of the filaments extend into its core.

In experiments performed by Yamada *et al.* (*21*) cytochalasin has been applied to elongating, dorsal root ganglion nerve cells (*22*). Cytochalasin causes microspikes to retract and growth cones to round up within 3 to 10 minutes. Axon elongation ceases as soon as these morphological changes occur (Fig. 5). Such an inhibited axon can remain quiescent for periods as long as 20 hours, during which time protein synthesis continues unabated (*23*).

The filamentous network of cytochalasin-arrested axonal tips is unusually "dense" and fails to show the normal polygonal pattern of the network found in active growth cones. In addition, an oriented anisotropic pattern of the network characteristic of active microspikes is also absent from these rounded axon tips. Microtubules, neurofilaments, and other organelles of the axon and growth cone appear unaffected by the drug.

When cytochalasin is removed from the culture medium, the rounded growth cone flattens within one to several hours (*24*) and microspikes appear. Axon elongation begins concurrently. This recovery still occurs under conditions in which 96 percent of protein synthesis is inhibited by $7 \times 10^{-5} M$ cycloheximide. In fact, dorsal root ganglion nerve cells will elongate for 10 to 24 hours under such conditions, even though the axon may become quite attentuated. These results indicate that no new protein synthesis need occur during the resumed elongation. Whatever proteins are required for such elongation either were manufactured during the period of cytochalasin arrest or may be derived from preexisting parts of the nerve cell (as, microtubule monomers from assembled microtubules) (*25*).

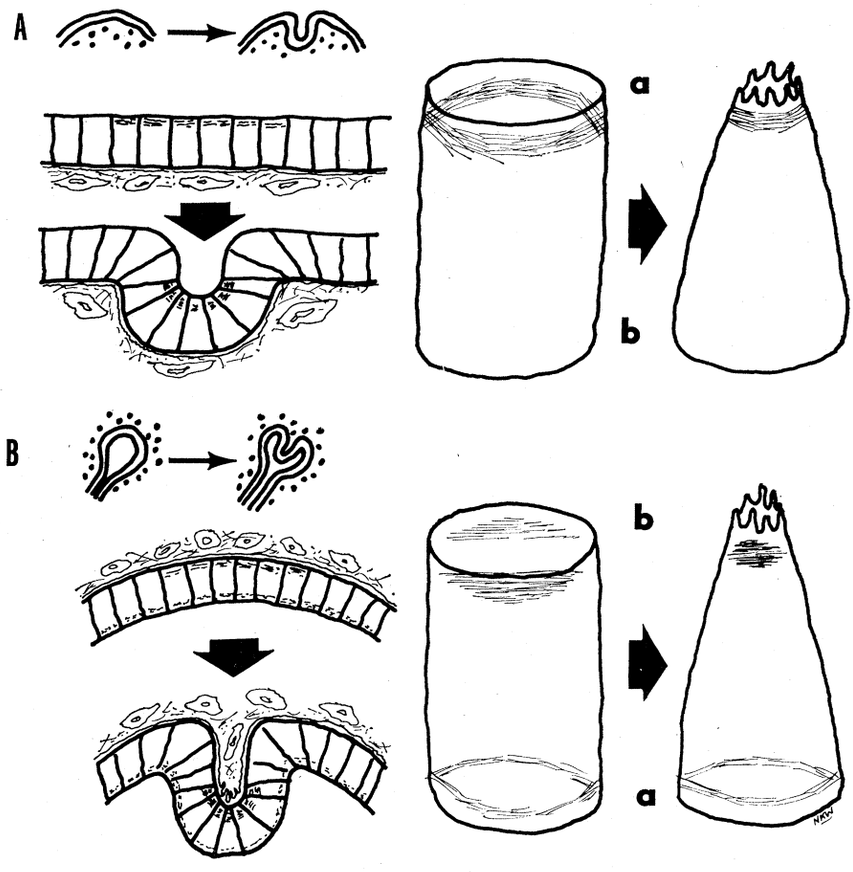

Fig. 1. Morphogenetic "movements" in epithelia in which a flat sheet of cells bulges inward, into mesenchyme (A) or outward, away from the mesenchyme and into a lumen (B). In (A) a purse string of microfilaments (*a*) is thought to contract to narrow the outer ends of cells and so cause the tissue movement. In (B) a purse string is also present at the luminal end (*a*) of the cells, but the critical contraction occurs at the opposite basal end (*b*) where bands or bundles of microfilaments are found.

It is particularly informative to contrast the effects of cytochalasin on nerves with those of colchicine. When the latter drug ($2.5 \times 10^{-6}M$, a concentration that is sufficient to disrupt nearly all microtubules of nerve and glial cells) is added to the medium, growth cone and microspike activity as well as elongation of the axon continue for about 30 minutes. The axon then begins to shorten, sometimes with bulging of its sides. Even as the axon retracts, the growth cone region and microspike activity appear unaffected. Ultimately axonal shortening reaches a point where the tip region is pulled free of the substratum; the growth cone then rounds up, and the whole axon sinks back into the nerve-cell body.

The conclusion from such observations is that there are two essential elements for axon elongation. First, the microtubule system serves as a skeletal system in the axon and its integrity is essential to the stability of the elongated structure. Elongation per se is dependent upon the growth cone–microspike system; presumably that organelle functions as an undulating membrane locomotory system, like that seen in typical migratory cells (*26*). If function of the microfilament network is disrupted with cytochalasin, outgrowth cannot take place despite the fact that protein synthesis continues and the microtubule skeleton is intact.

Single-Cell Movement

Both the halt in migration of fibroblasts (*2, 13, 14*) and the cessation of growth cone locomotory function caused by cytochalasin, suggest that the hypothesized contractile machinery used in cell movement (*27*) is sensitive to cytochalasin. In experiments performed by Spooner *et al.* (*28*), glial cells from dissociated dorsal root ganglia have been subjected to cytochalasin and colchicine treatment. Heart fibroblasts have been analyzed similarly by Taylor and Wessells (*29*). Untreated cells of both types cultured in low density possess typical undulating membrane organelles (*26*) that contain a network of microfilaments (Fig. 2C) resembling that seen in growth cones of nerve cells. In addition, a thin sheath of microfilaments lies inside the plasma membrane of fibroblastic and glial cells, and in both cases most of the filaments run parallel to the long axis of the cells (that is, to the direction of cell movement). Thickened portions of the same sheath extend into the long processes characteristic of glia and fibroblasts in cell culture (*30*). These sheath filaments probably correspond to the cortex filaments of chondrocytes that can bind heavy meromyosin, and in this respect can be thought of as a kind of actin (*31*).

When exposed to cytochalasin (7 μg/ml) glial cells stop moving in about 10 to 15 minutes and at later times begin to assume stellate shapes in which long processes are left protruding as straight, stiff rods from the central cell body (this occurs because intervening regions of cell surface sink back toward the cell body). Heart fibroblasts also halt within 15 minutes and generally assume a similar form. When the ultrastructure is examined, the sheath of filaments 50 Å in diameter in both cell types is intact and the thick bundles of similar filaments still extend down the core of the elongate cell processes. However, the filamentous network near the plasma membrane is altered by cytochalasin (Fig. 3B), and initial changes are observed as soon as 8 minutes after the drug is applied. Large masses of granular and fine filamentous material are found beneath the membrane as well as in internal regions of the cells. These masses are like those seen in cytochalasin-treated epithelial cells of oviduct

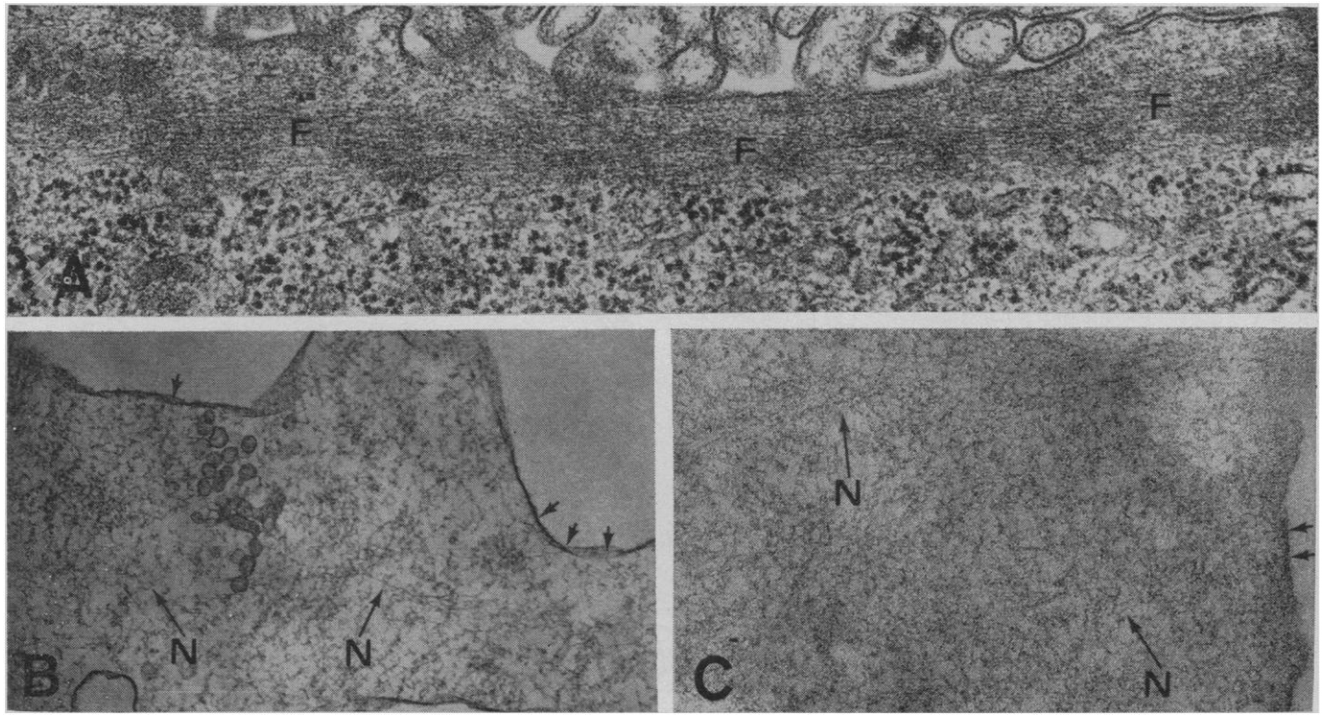

Fig. 2. The two basic types of putative contractile systems. In (A) a band of microfilaments (*F*) is seen extending across the luminal cytoplasm of an estrogen-stimulated, chick oviduct cell. Individual filaments average 40 to 60 Å in diameter. Note that ribosomes and other organelles are excluded from the band of filaments. Microfilament networks (*N*) are seen in the growth cone of an elongating axon (B) and of a migratory glial cell (C). The network inserts on the plasma membrane (arrows) and shows asymmetries in shape in various regions. Networks in migratory heart fibroblasts are identical to the ones pictured here.

and salivary gland. Thus there appear to be two populations of 50-Å filaments in these migratory cells. The network is sensitive to cytochalasin, whereas the sheath is not. The undulating membrane stops functioning, and cell movement ceases when the network is disrupted by the drug.

Concentrations of colchicine that result in the disappearance of all cytoplasmic and spindle microtubules from glial cells do not disrupt either of the 50-Å filament systems, nor do these concentrations alter visible function of the undulating membrane or inhibit cell movement (*28*). The continued function of the locomotory apparatus, despite disruption of microtubules, corresponds to the persisting activity of axonal growth cones in colchicine. Such axonal growth cones cease functioning only after retraction of the axon reaches the point of disrupting the relation between growth cone and substratum. There, the effects of colchicine upon the locomotory organelle are secondary; in glia and heart fibroblasts no effects have been demonstrated.

These observations have led to a proposal (*28*) that the essential components of the undulating membrane are a contractile microfilament network and the plama membrane. The filamentous sheath might serve as a relatively stiff but bendable skeleton that could prevent gross shortening of the cell when the filament network functions during movement of areas of the undulating membrane. Or, if its capacity to bind heavy meromyosin (*31*) means that it functions like actin, the sheath may form a rigid base against which the contractile filamentous network can act to generate movement of the cell surface. The presence of smooth endoplasmic reticulum both in the undulating membrane and in the axonal growth cone further suggests that addition of new membrane at the anterior edge of the cell may be a normal part of locomotory activity (*28, 32*). It seems clear that further understanding of the cytochalasin-sensitive network as a contractile apparatus and in relation to the plasma membrane and filamentous sheath is the key to our understanding of the fundamental capacity of cells to move over a substratum.

Cytoplasmic Streaming

Rotational streaming of the peripheral cytoplasm occurs in many plant cells. Microfilaments with a diameter of

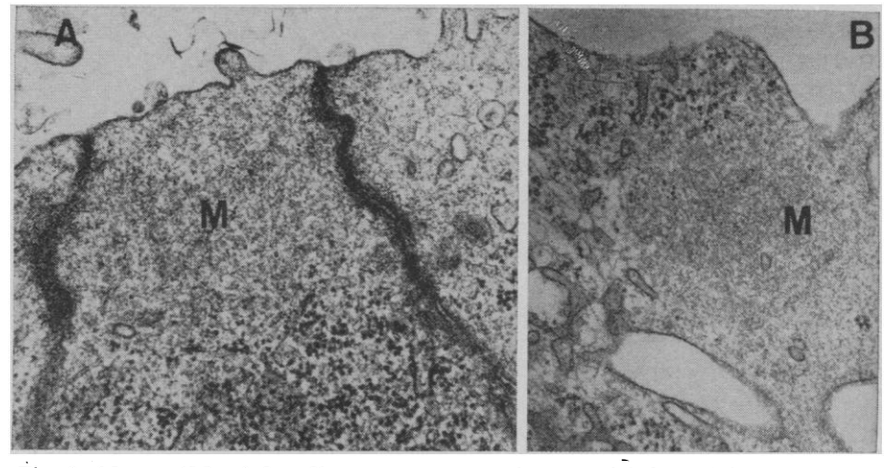

Fig. 3. Masses (*M*) of fine filamentous or granular material formed in response to cytochalasin in an oviduct (A) and a glial (B) cell. A band, as seen in Fig. 2A, formerly extended across this portion of the oviduct cell, and a loose network (as in Fig. 2, B and C) was present in this part of the glial cell. The drug disrupts both of those systems and the masses are found instead.

50 Å are located near the stream and are thought to provide the motive force for streaming (*33*). In order to test this possibility, Bradley (*34*) has applied cytochalasin to the large, single internode cells of the alga *Nitella* and to the smaller cells of the oat (*Avena*) coleoptile.

Cytochalasin (30 μg/ml) completely stops cytoplasmic streaming within 1 hour in both *Nitella* (Fig. 6) and *Avena* cells. If the cytochalasin is washed out of the medium, both cell types quickly begin vigorous streaming again. The complete recovery to the initial rates (Fig. 6) indicates that no permanent damage is done by the drug treatment.

As in the other systems so far tested, protein synthesis appears to be unnecessary for the recovery of the biological process after cytochalasin treatment. The continued presence of 10 μg of cycloheximide per milliliter for 17 hours before cytochalasin treatment, during treatment, and after treatment was stopped allowed the initial streaming rates in *Nitella* internodes to appear again (Fig. 6). Likewise in *Avena*, a 1-hour preliminary incubation in cycloheximide (10 μg/ml) did not prevent the recovery of vigorous streaming. It is remarkable that the long-term maintenance and the functional reassembly of the streaming apparatus are so independent of protein synthesis.

Colchicine has been used by itself or in combination with cytochalasin to assess the role of microtubules in streaming. In both *Avena* and *Nitella*, streaming continues unabated in the presence of $10^{-2}M$ colchicine, which is known to disrupt plant cytoplasmic microtubules (*35*). If either cell type is incubated in $10^{-2}M$ colchicine 2 hours before, during, and after treatment with cytochalasin, no effects are detectable; streaming is stopped by cytochalasin and starts again after the drug is removed.

In summary, the presence of microfilaments in cells that stream, the inhibition of streaming by cytochalasin, and the known effects of the drug on similar microfilament systems in animal cells allow us to predict that microfilaments are the causal agents of streaming in plant cells.

Other Biological Effects of Cytochalasin

Cytochalasin has been applied to a number of cell systems thought on independent grounds to be contractile. Tunicate tadpole tail shortening has been investigated (*36*) by R. A. Cloney in *Distaplia*, by J. Lash in *Amaroucium*, and by M. O. Bradley in *Ciona*. When the drug was added to tadpoles at any time during tail resorption (*37*), it causes rapid cessation of the shortening process. As compared to controls, treated cells show disruption of the microfilament bands in both *Distaplia* and *Ciona*. As noted by Cloney, this effect is most obvious in central regions of the cells treated with low doses of cytochalasin, whereas some intact filaments may continue to insert near the plasma membrane. These effects are reversible, so that tail shortening starts again soon after removal of cytochalasin from the seawater.

Cytochalasin has also been applied

by Spooner and Wessells to minute explants of cardiac muscle from 11-day mouse embryos grown in organ culture [as in (*13*)], and by Taylor and Wessells (*29*) to dissociated, beating, single heart cells from 8-day chick embryos (*38*). When cytochalasin is applied, beating gradually decreases in frequency and halts completely by 4 to 6 hours. In the same cell cultures, heart fibroblasts round up and stop migratory activity in 10 to 15 minutes. The heart fibroblasts can recover and move again; this occurred even in cells that by mistake were kept for 7 days in cytochalasin. In contrast, heartbeat has not resumed in any cell or organ culture, although heart myoblasts do spread "thin" upon the substratum after cytochalasin is removed. Initial electron microscopic observations have revealed that a variable percentage of the sarcomeres in cytochalasin-treated cells are arranged at abnormal angles to one another and that Z bands are infrequent in many cells. Both thick myosin and thin actin filaments are present, though such cells show no continued ability to contract spontaneously. Therefore, in inhibiting cardiac contractions, cytochalasin does not cause a wholesale disruption of that portion of the contractile machinery.

Smooth muscle differs from cardiac muscle in that it does reverse after cytochalasin is removed. Spooner has applied the drug to primitive mouse embryonic guts that developed spontaneously contracting smooth muscle in vitro (*11*). Such peristaltic contractions cease about 4 hours after drug application. Then, after as long as 24 hours of inhibition, removal of the drug is followed by resumed peristalsis. In fact, the same cultures can be stopped and started again by another course of cytochalasin treatment. Contractions of muscle that develop in the wall of the gut of *Urechis caupo* trochophore larvae is also reversibly inhibited by 10 μg of cytochalasin per milliliter (*39*).

The basis for the difference between heart and smooth muscle cells in recovery from cytochalasin is unknown, although the contractile filament bundles of smooth muscle are relatively unordered in comparison with the sarcomeres of cardiac muscle. Perhaps the more complex architectural arrangement in heart muscle that arises over a relatively long developmental period cannot be reconstituted functionally after cytochalasin removal, whereas the simpler thin filaments of smooth muscle can function once more.

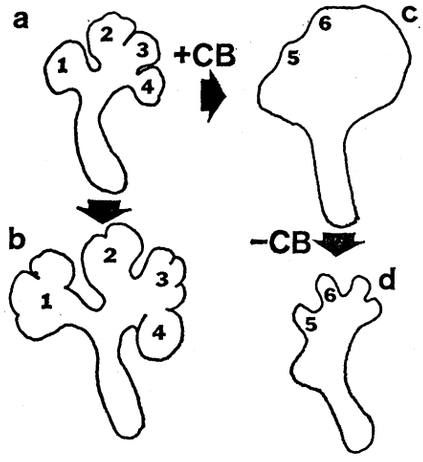

Fig. 4. Tracings of Kodachromes of living salivary epithelia. A control epithelium after 24 (a) and 48 (b) hours of organ culture. Compare regions 1 through 4 at the two times to envision the progressive branching that occurs in this tissue. Eighteen hours after addition of cytochalasin a tissue similar to that seen in (a) would apper as in (c); 24 hours after the drug was removed (d), the tissue has rounded up and clefts have reappeared (as between No. 5 and No. 6, and so forth).

After the blood in higher vertebrates undergoes the initial clotting reaction a process of clot retraction takes place (*40*). This phenomenon has been attributed to the contractile protein thrombosthenin located within the blood platelets. Thrombosthenin is present as filaments, 80 to 100 Å in diameter, within platelets (*41*). In experiments performed by Yamada and Taylor (*42*), fresh citrated whole blood from roosters or from samples provided by Dr. Donald Kennedy or platelet-rich plasma was clotted (*43*). Normal clots retracted during the subsequent 0.5 to 18 hours of incubation at 37°C. Clots also formed and retraction occurred in the presence of dimethylsulfoxide, or colchicine, even though the blood was first incubated in these agents, that is, prior to initiation of clotting (*44*). However, if the initial incubation was done in cytochalasin, a clot formed with normal speed but did not retract. The clot remained in close proximity to the vessel wall, and no serum was expressed. Cytochalasin has no effect when platelet aggregation, an early step in normal clot formation, is induced by adenosine diphosphate. As seen in the electron microscope, cytochalasin-treated platelets were most often rounded up and possessed relatively few pseudopods. Masses of densely packed material, like those present in treated migratory and epithelial cells, are seen, whereas a loosely spaced filamentous network, characteristic of control platelets, is absent. This alteration in the microfilament system can be correlated with the inability of clots to retract and is consistent with the hypothesis that the microfilament system of platelets is the contractile organelle. These and other observations, such as the ability of platelets to spread on a glass substratum, suggest strongly that a platelet behaves much like an undulating membrane organelle of a migratory cell.

Cytochalasin has also been used as a diagnostic probe on a system that has not yet been examined for microfilament arrays. The flat vegetal plate of many marine embryos undergoes an invagination process during gastrulation which is nearly identical in form to invagination of the lens placode, a process thought to be dependent upon microfilament purse strings (*7*). Luduena (*39*) has shown that cytochalasin stops invagination in embryos of sea urchins, a sand dollar, and an echiuroid worm (*45*). Thus, embryos treated before invagination starts fail to initiate that process; those treated as early or midgastrulae cease invaginating very soon after cytochalasin is added (*46*). Recovery from such inhibition is variable: some *Strongylocentrotus, Urechis,* and *Dendraster* embryos invaginate again, whereas others exogastrulate (*47*). The inhibition by cytochalasin is probably not an indirect effect of contractile ring malfunction (although that, no doubt, is occurring), since an antimitotic dose of Colcemid (*48*) does not prevent invagination or cause exogastrulation in *Dendraster.* Since some 50-Å filament bundles have been observed in embryos related to those used here (*49*), it is clearly necessary to examine vegetal plates in order to determine whether the inhibition of invagination by cytochalasin is due to disruption of filaments.

Gingell has described calcium-activated contractions in the cortex of amphibian eggs (*50*). Ash (*51*) has repeated and confirmed those experiments involving direct injection of calcium beneath the cell membrane of *Xenopus laevis* embryos (*52*). Such injections initiate cortical contractions that can be seen because pigment granules are drawn into a black ring around the electrode tip. Microfilaments, probably arrayed as a network, are seen at such injection sites. After cytochalasin has been applied, the capacity of the cell to carry out this contraction dimin-

ishes, and after 20 minutes of treatment, calcium no longer can initiate contraction. Concomitantly, the ability of cell cortex to show wound healing (*50*) is also lost. Since microfilaments have been reported near the surface of various egg types (*4, 50*), and since cytochalasin's only known action is upon microfilaments, these experiments suggest that the calcium-activated contractile system involves such filaments.

Summary and Conclusions

In our opinion, all of the phenomena that are inhibited by cytochalasin can be thought of as resulting from contractile activity of cellular organelles. Smooth muscle contraction, clot retraction, beat of heart cells, and shortening of the tadpole tail are all cases in which no argument of substance for alternative causes can be offered. The morphogenetic processes in epithelia, contractile ring function during cytokinesis, migration of cells on a substratum, and streaming in plant cells can be explained most simply on the basis of contractility being the causal event in each process. The many similarities between the latter cases and the former ones in which contraction is certain argue for that conclusion. For instance, platelets probably contract, possess a microfilament network, and behave like undulating membrane organelles. Migrating cells possess undulating membranes and contain a similar network. It is very likely, therefore, that their network is also contractile.

In all of the cases that have been examined so far, microfilaments of some type are observed in the cells; furthermore, those filaments are at points where contractility could cause the respective phenomenon. The correlations from the cytochalasin experiments greatly strengthen the case; microfilaments are present in control and "recovered" cells and respective biological phenomena take place in such cells; microfilaments are absent or altered in treated cells and the phenomena do not occur. The evidence seems overwhelming that microfilaments are the contractile machinery of nonmuscle cells.

The argument is further strengthened if we reconsider the list of processes insensitive to cytochalasin (Table 2). Microtubules and their sidearms, plasma membrane, or synthetic machinery of cells are presumed to be responsible for such processes, and

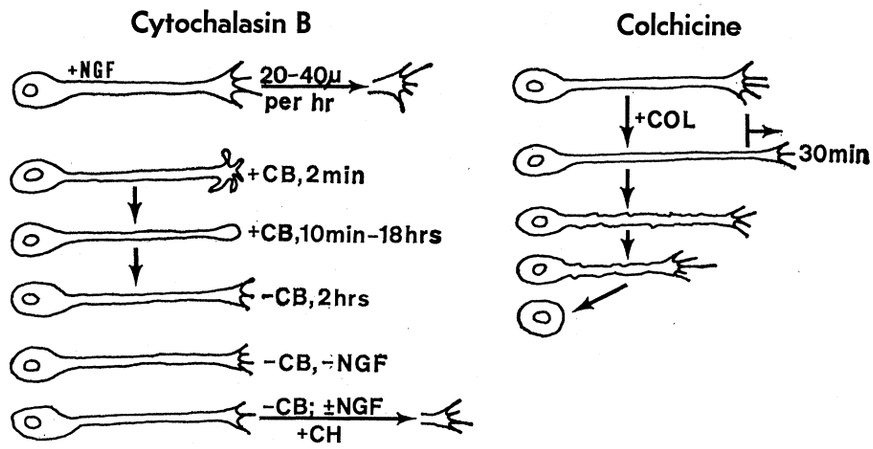

Fig. 5. A summary of experiments on elongating axons of spinal ganglion cells (*21*). Cytochalasin halts growth cone function and elongation, but the effect is fully reversible even in the absence of exogenous nerve growth factor (*NGF*) or presence of cycloheximide (*CH*). Colchicine only inhibits elongation indirectly after the axonal tip pulls free of the substratum.

colchicine, membrane-active drugs, or inhibitors of protein synthesis are effective at inhibiting the respective phenomena. These chemical agents would not necessarily be expected to affect contractile apparatuses over short periods of time, they either do not or only secondarily interfere with the processes sensitive to cytochalasin (Table 1). It is particularly noteworthy in this context that microtubules are classed as being insensitive to cytochalasin and so are not considered as members of the "contractile microfilament" family.

The overall conclusion is that a broad spectrum of cellular and developmental processes are caused by contractile apparatuses that have at least the common feature of being sensitive to cytochalasin. Schroeder's important insight (*3*) has, then, led to the use of cytochalasin as a diagnostic tool for such contracile activity: *the prediction is that sensitivity to the drug implies presence of some type of contractile microfilament system.* Only further work will define the limits of confidence to be placed upon such diagnoses.

The basis of contraction in microfilament systems is still hypothetical. Contraction of glycerol-extracted cells in response to adenosine triphosphate (*53*), extraction of actin-like or actomyosin-like proteins from cells other than muscle cells (*54*), and identification of activity resembling that of the actomyosin-adenosine triphosphatase system in a variety of nonmuscle tissues (*40, 54*) are consistent with the idea that portions of the complex, striated muscle contractile system may be present in more primitive contractile machinery. In the case of the egg cortex, calcium-activated contractions can be inhibited by cytochalasin. If, as seems likely, microfilaments are the agents activated by calcium, then it will be clear

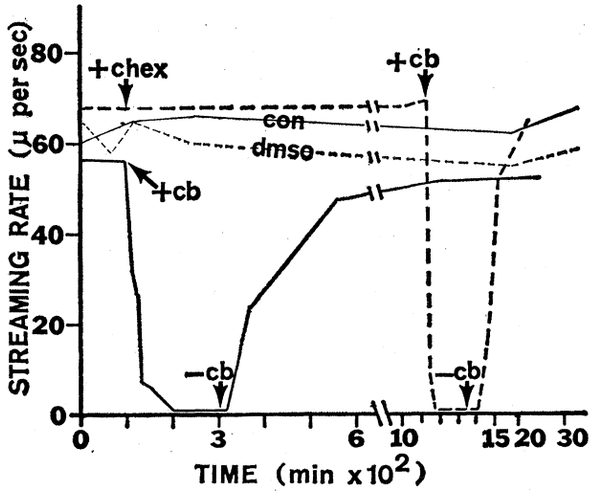

Fig. 6. The rate of cytoplasmic streaming in *Nitella* internode cells as a function of time. Data from four typical cells is shown: Control (*con*) and dimethylsulfoxide (*dmso*) streaming is identical when many cells are compared. Addition of cytochalasin (*cb*) results in rapid cessation of streaming, and reinitiation occurs after drug removal (*–cb*), even in the presence or absence of cycloheximide (*chex*).

that they have the same calcium requirement as muscle.

Biochemical analyses of primitive contractile systems are difficult to interpret. Ishikawa's important observation (*31*), that heavy meromyosin complexes with fine filaments oriented parallel to the surface of chondrocytes and perpendicular to the surface of intestinal epithelial cells, implies that both types of filaments are "actin-like" in this one respect. Yet, it is very likely that these actin-like filaments correspond respectively to the *cytochalasin-insensitive* sheath of glial and heart fibroblasts and the core filaments of oviduct microvilli. No evidence from our studies links contractility directly to these meromyosin-binding filaments. Apart from this problem, activity resembling that of the myosin-adenosine triphosphatase has been associated with the microtubule systems of sperm tails and cilia (*55*), but those organelles are insensitive to cytochalasin in structure and function. Clearly, a means must be found to distinguish between enzymatic activities associated with microfilament networks, microfilament bundles, microtubules, and the sheath filaments of migratory cells. Until such distinctions are possible, little of substance can be said about the molecular bases of primitive contractile systems.

Three variables are important for the control of cellular processes dependent upon microfilaments: (i) which cells of a population shall manufacture and assemble the filaments; (ii) where filaments shall be assembled in cells; and (iii) when contractility shall occur.

With respect to distribution among cells, the networks involved in cell locomotion are presumed to be present in all cells that have the potential to move in cell culture. In this respect, the networks can be regarded as a common cellular organelle in the sense that cytoplasmic microtubules are so regarded. In some developing systems, all cells of an epithelium possess microfilament bundles (*7, 13*), whereas, in others, only discrete subpopulations possess the bundles (*5, 6*). In these cases the filaments can be regarded as being differentiation products associated only with certain cell types. These considerations may be related to the fact that microfilament networks are associated with behavior of individual cells (such as migration, wound healing, and cytokinesis), whereas the bundles are present in cells that participate in coordinated changes in shape of cell populations.

With respect to placement in cells, two alternatives are apparent, namely, localized or ubiquitous association with the plasma membrane. Microfilament bundles of epithelial cells are only found extending across the luminal and basal ends of cells. In this respect they contrast with desmosomal tonofilaments and with microtubules, each of which can curve in a variety of directions through the cell. The strict localization of microfilament bundles probably rests upon their association with special junctional complex insertion regions that are only located near the ends of cells. In the case of mitotically active cells, the orientation of the spindle apparatus may determine the site at which the contractile ring of microfilaments will form (*4, 56*); this raises the question of what sorts of cytoplasmic factors can influence the process of association between filament systems and plasma membranes.

In contrast to such cases of localized distribution, contractile networks responsible for cell locomotion are probably found beneath all of the plasma membrane, just as the network of thrombosthenin may extend to all portions of the periphery of a blood platelet. This ubiquitous distribution probably accounts for the ability of a fibroblast or glial cell to establish an undulating membrane at any point on its edge, or of an axon to form lateral microspikes along its length.

The third crucial aspect of control of these contractile apparatuses involves the choice of *when* contraction shall occur (and as a corollary the degree or strength of contraction that will occur). In the simplest situation, contraction would follow automatically upon assembly of the microfilament bundles or networks. In cleavage furrows of marine embryos (*4*), for instance, microfilaments are seen beneath the central cleavage furrow and at its ends, but not beyond, under the portion of plasma membrane that will subsequently become part of the furrow. This implies that the furrow forms very soon after the contractile filaments are assembled in the egg cortex.

In other cases, microfilaments are apparently assembled but not in a state of (maximal?) contraction. Thus, networks are seen along the sides of migratory cells, although such regions are not then active as undulating membrane organelles. Similarly, microfilament bundles occur in all epithelial cells of the salivary gland (*13*), or pancreatic anlage (*7*), although only the ones at discrete points are thought to generate morphogenetic tissue movements. Likewise, bundles begin to appear as early as 12 hours after estrogen administration to oviduct, although visible tubular gland formation does not start until 24 to 30 hours. Finally, streaming in plant cells can wax and wane, depending upon external factors such as auxin (*57*). All of these cases imply a control mechanism other than mere assembly of the microfilament systems and even raise the possibility that within one cell some filaments may be contracting while others are not.

In discussing this problem, it must be emphasized that different degrees of contraction or relaxation cannot as yet be recognized with the electron microscope. In fact, every one of the cases cited above could be explained by contraction following immediately upon some subtle sort of "assembly." Inclusive in the latter term are relations between individual filaments, relations of the filaments and their insertion points on plasma membrane, and quantitative alterations in filament systems. Furthermore, the critical role of calcium and high-energy compounds in muscle contraction suggest that equivalent factors may be part of primitive, cytochalasin-sensitive systems. The finding that calcium-induced contraction in the cortex of eggs is sensitive to cytochalasin strengthens that supposition and emphasizes the importance of compartmentalization of cofactors as a means of controlling microfilaments in cells.

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14. The mesenchymal cells surrounding such epithelia fail to migrate as individuals away from the main mesenchymal mass as long as cytochalasin is present (see below for further discussion).

15. Dimethylsulfoxide (DMSO) (1 percent), the carrying solubilizer of cytochalasin, has no effect on the morphogenesis of control salivary epithelia, nor does it affect any of the other biological processes to be discussed below (nerve elongation, cell locomotion, heart cell beating, and other). DMSO does not decrease incorporation of labeled amino acid into hot, acid-insoluble material. Furthermore, cytochalasin does not depress such protein synthesis in glial, nerve, and salivary gland cells.
16. In such regions at the base of clefts, the basal surface of the cells is highly twisted and folded; and the overlying basal lamina is often thrown free of its normal close association with the plasma membrane of the epithelial cells. Analogous foldings and convolutions have been reported in every other case in which purse-string activity is thought to account for rapid decreases in the cross-sectional area of cells (Fig. 1).
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22. Since such nerve cells do not divide, these experiments eliminate the possibility that all cytochalasin effects are indirect results of inhibition of cytokinesis.
23. Grain counts over autoradiograms show no statistically significant (1 percent) difference between label incorporated by single cytochalasin-arrested cells compared to controls.
24. The time for recovery is dependent on the dose of cytochalasin that caused rounding up.
25. A similar phenomenon operates in nerve cells kept in suspension culture or under certain nutrient conditions. In the former case, no axons can form, probably because of unavailability of a substratum for the growth cone. In the latter case, a change in serum concentration permits outgrowth even if protein synthesis is inhibited [J. Olmstead, K. Carlson, R. Klebe, F. Ruddle, J. Rosenbaum, *Proc. Nat. Acad. Sci. U.S.* **65**, 129 (19' → N. W. Seeds, A. G. Gilman, T. Amano, M. W. Nirenberg, *ibid.* **66**, 160 (1970)]. Reversal from cytochalasin occurs in medium from which nerve growth factor is omitted, and normal axon elongation takes place. [See also R. Levi-Montalcini, *Science* **143**, 105 (196 → S. Cohen, *Proc. Nat. Acad. Sci. U.S.* **46**, 302 (1960)].
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37. Used at 0.25 to 10 μg/ml in seawater.
38. Eight-day embryonic chick ventricle was dissociated with 0.25 percent trypsin in Ca- and Mg-free Tyrode solution, 37°C, 60 minutes; 2×10^5 cells were plated in plastic petri dishes in Eagle's minimum essential medium containing bovine serum (16 percent) and chick embryo extract (2 percent), and incubated at 37°C. Either cytochalasin or DMSO was added after 24 hours of incubation.
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43. Ca^{++} (final concentration $10^{-2}M$) and a small portion of juice from chick embryos were added to initiate clotting.
44. DMSO (1 percent); colchicine ($2.5 \times 10^{-6}M$); cytochalasin (10 μg/ml). Preliminary incubation for 30 minutes at 37°C.
45. *Strongylocentrotus purpuratus, Urechis caupo* (10 μg/ml); *Lytechinus pictus, Dendraster excentricus* (3 μg/ml). Recovery is a function of the stage at which cytochalasin is applied, and the length of time that the drug was present.
46. Ciliary function is not affected in *Strongylocentrotus* and *Urechis*, although embryos rotate instead of swimming along straight paths. Many cilia are lost from the surfaces of *Lytechinus* and *Dendraster* embryos, an observation of interest because cilia are apparently "anchored" by filament bundles [D. W. Fawcett and K. R. Porter, *J. Morphol.* **94**, 221 (1954)].
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59. *Escherichia coli* C600 growth rates were measured in exponential cultures by increase in optical density; cell motility and morphology were observed under phase contrast microscopy. Cultures receiving 10 μg of cytochalasin B per milliliter or 1 percent DMSO were normal. After prior incubation in cytochalasin B (50 μg/ml), *E. coli Hfr H* gal^+ str^s was mated for 90 minutes with a $gal^-F^-str^r$ recipient in tryptone broth without shaking. Frequency of gal^+ recombinants measured on galactose streptomycin plates was identical in control, 1 percent DMSO, or cytochalasin cultures. These experiments were performed by Gary Ketner.
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