Action of Cytochalasin D on Cytoskeletal Networks

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ABSTRACT   Extraction of BSC-1 cells (African green monkey kidney) with the detergent Triton X-100 in combination with stereo high-voltage electron microscopy of whole mount preparations has been used as an approach to determine the mode of action of cytochalasin D on cells. The cytoskeleton of extracted BSC-1 cells consists of substrate-associated filament bundles (stress fibers) and a highly cross-linked network of four major filament types extending throughout the cell body: 10-nm filaments, actin microfilaments, microtubules, and 2- to 3-nm filaments. Actin filaments and 2- to 3-nm filaments form numerous end-to-side contacts with other cytoskeletal filaments. Cytochalasin D treatment severely disrupts network organization, increases the number of actin filament ends, and leads to the formation of filamentous aggregates or foci composed mainly of actin filaments. Metabolic inhibitors prevent filament redistribution, foci formation, and cell arborization, but not disorganization of the three-dimensional filament network. In cells first extracted and then treated with cytochalasin D, network organization is disrupted, and the number of free filament ends is increased. Supernates of preparations treated in this way contain both short actin filaments and network fragments (i.e., actin filaments in end-to-side contact with other actin filaments). It is proposed that the dramatic effects of cytochalasin D on cells result from both a direct interaction of the drug with the actin filament component of cytoskeletal networks and a secondary cellular response. The former leads to an immediate disruption of the ordered cytoskeletal network that appears to involve breaking of actin filaments, rather than inhibition of actin filament-filament interactions (i.e., disruption of end-to-side contacts). The latter engages network fragments in an energy-dependent (contractile) event that leads to the formation of filament foci.

The cytochalasins have now been used for more than a decade as tools to study motility-related phenomena in a variety of cell types (e.g., references 7, 11, 26, 27, 39; for an overview, see reference 33). They exert a number of dramatic morphological effects on cells, including inhibition of ruffling and motility, cell retraction and arborization, zeiosis, blebbing, and enucleation (reviewed in reference 14). Because of their profound effects on cell morphology and motility, and because of numerous electron microscope observations of marked effects on filamentous structures, it had early been assumed that their primary site of action is the cellular contractile machinery. In fact, cytochalasin-sensitivity alone has often been taken as an indication for the involvement of an actin-based contractile system in certain cellular activities, even in the absence of additional supportive morphological or physiological evidence.

However, the mechanism of action of cytochalasin has begun to be understood only recently. In vitro studies using purified actin clearly demonstrate inhibition of the rate of actin filament polymerization (5, 8, 23, 24) which seems to be due to inhibition of actin subunit addition to the fast-growing or "barbed" end of actin filaments (9, 23, 24). This inhibitory action apparently involves binding to high affinity binding sites or F-actin (5, 6, 8, 12). Several reports also demonstrated profound effects of cytochalasins on gels of cytoplasmic extracts (e.g., references 29, 38), mixtures of purified gelation factors and actin (18, 19, 24), or microtubules and actin filaments (17). In addition, cytochalasins were shown to reduce the low-shear viscosity of actin filaments alone (22, 24). On the basis of the evidence indicating binding of cytochalasin B to actin filament ends, MacLean-Fletcher and Pollard (24) and Lin and Lin (22) proposed that binding of cytochalasin to the fast-growing end of actin filaments also explains its inhibitory action on network formation. Hartwig and Stossel (19), on the other hand, suggest that the cytochalasins interfere with network structures formed

by actin filaments and a gelation factor (actin-binding protein) by severing or cleaving actin filaments at limited sites, thereby reducing actin filament length.

The relationship of these in vitro effects to the dramatic in vivo changes is, however, not clear. This study analyzes cytochalasin-induced cytoskeletal changes using, as a methodological approach, a combination of detergent extraction, whole mount preparation, and stereo high-voltage electron microscopy. It was hoped that the now widely used method of cell extraction to study cytoskeletal organization (e.g., references 2, 10, 21, 28, 31, 37) will provide some insights into the effects of this drug on cell organization. This approach was used here to analyze the effects of cytochalasin D on cellular filament networks.

MATERIALS AND METHODS

Cells

African green monkey kidney cells (strain BSC-1) were used for the experiments described here. They were grown in Dulbecco's medium supplemented with 10% fetal calf serum. For experiments, cells were seeded in 35-mm plastic tissue culture dishes containing gold grids sandwiched between a glass cover slip and a Formvar film (31, 40).

Detergent Extraction and Fixation

The buffer used in extraction experiments consisted of 60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9 (PHEM buffer). Cells grown on gold grids were briefly washed with PHEM buffer, extracted with 0.1% Triton X-100 in PHEM buffer for 1-2 min, and thoroughly washed with PHEM buffer. Cells were then fixed with 1% glutaraldehyde in PHEM buffer containing 0.2-0.5% tannic acid for 25 min, washed with buffer, postfixed with 0.5% OsO_4, and processed for high-voltage electron microscopy.

Heavy Meromyosin-Subfragment I Decoration

Heavy meromyosin-subfragment (S1) was kindly provided by Dr. Frank Pepe (University of Pennsylvania). After Triton X-100 extraction, cover slips were immersed in S1 (~1 mg/ml) in PHEM buffer supplemented with 15% glycerol for 1-5 min. Cells were then thoroughly washed and fixed as described above.

Drug Treatments

Cytochalasin D was obtained from Sigma Chemical Co. (St. Louis, Mo.) and was kept as a stock solution of 1 mg/ml in dimethyl sulfoxide (DMSO) at −20°C. It was applied to cells at final concentrations of 1-5 μg/ml for various periods of time (1-60 min). Control preparations received 0.5% DMSO.

In another set of experiments, cells grown in 60-mm petri dishes were first extracted with Triton X-100 for 1-2 min, washed twice with PHEM buffer, and then treated with 2 μg/ml cytochalasin D in PHEM buffer for 1-15 min. Control preparations received 0.2% DMSO. The supernates of these preparations were either used for negative staining or processed for PAGE. The same protocols were also used for cells grown on gold grids. These preparations were fixed and processed for high-voltage electron microscopy.

Inhibitors of energy metabolism were dissolved in Hank's balanced salt solution at the following final concentrations: 2-deoxyglucose 10 mM, dinitrophenol 1 mM, oligomycin 10 μg/ml. The latter two agents were used either alone or in combination with 2-deoxyglucose. Cells grown on gold grids were pretreated with inhibitors for 20-40 min and then exposed to cytochalasin D or DMSO for up to 30 min in their continued presence.

Actomyosin Preparation

Actomyosin was prepared from rabbit back and leg muscle according to the procedure of Beck et al. (1), with slight modifications. About 100 g of muscle tissue were minced and grinded by three strokes of 15 sec each in a Waring blender (Waring Products Div., Dynamics Corp. of America, New Hartford, Conn.) in a buffer containing 0.6 M KCl and 0.01 M phosphate buffer, pH 7.0 (high ionic strength buffer). This mixture was stirred overnight at 4°C and centrifuged at 10,000 g for 30 min. The supernate was diluted to 0.06 M KCl, 0.01 M phosphate buffer (low ionic strength buffer), and the precipitated actomyosin was pelleted at 10,000 g. The actomyosin was further purified by two

more cycles of high ionic strength dissolution–low ionic strength precipitation, made 40% glycerol in high ionic strength buffer, and stored at −20°C.

Actomyosin droplets were produced by spraying an actomyosin solution in high ionic strength buffer through a glass pipette (tip diameter ~1 mm) onto the surface of a Formvar and carbon-coated 40 mesh copper grid. The grids were immediately plunged into low ionic strength buffer to precipitate the actomyosin. Some grids were further treated with 2 mM ATP in low ionic strength buffer for 2 min to induce actomyosin contraction (superprecipitation). Grids with both precipitated and superprecipitated actomyosin were fixed with 1% glutaraldehyde in low ionic strength buffer, postfixed with OsO_4, and processed for whole mount high-voltage electron microscopy.

Protein Determination

Protein concentrations were determined according to the procedure described by Bradford (4).

Negative Staining

400 mesh copper grids were prepared for negative staining by coating with a Formvar film onto which a thin film of carbon was evaporated briefly before the experiment. Supernates of cytochalasin D or DMSO-treated cytoskeletons were applied to the grids, blotted, washed with PHEM buffer and negatively stained with a freshly prepared 1% solution of uranyl acetate.

Gel Electrophoresis

PAGE was performed on 7.5% gel slabs in the presence of mercaptoethanol and SDS according to the procedure of Laemmli (20).

Light Microscopy

Phase-contrast microscopy was performed on cells growing on cover slips using a Zeiss light microscope equipped with a × 40 phase-contrast objective.

High-voltage Electron Microscopy

After fixation (see above), cells were dehydrated in ethanol, and dried from CO_2 according to the critical point method. The preparations received a thin film of carbon to increase stability before viewing in a JEOL 1,000,000 V microscope. Stereo micrographs were generated at tilt angles of ± 4-12°.

RESULTS

Cytochalasin D Applied to Intact Cells

Treatment of BSC-1 cells with 2-4 μg/ml cytochalasin D, a congener that does not affect sugar transport (35), induces the well-known morphological changes described for a variety of cell types: inhibition of ruffling, formation of phase-dense aggregates or "foci," first in the cell periphery and later (after ~5 min) throughout the cell body, dissolution of stress fibers, cell arborization (which commences after ~10-15 min of treatment), and rounding up of the perinuclear cell area (for an overview, see reference 14). Prolonged (>30 min) treatment results in pronounced cell arborization (Fig. 1). For the electron microscopy studies described here, most cells were used after a 15-20 min treatment with cytochalasin D (see Fig. 1), at which time they showed pronounced foci formation and stress fiber dissolution, but only moderate or beginning arborization. These cells are well suited for stereo electron microscopy and yet display all the characteristics of cytochalasin treatment.

Morphological changes in whole, unextracted critical point–dried cells have already been studied by Fonte et al. (13) and will not be described here. This analysis concentrates on effects on the filament network as seen after detergent extraction and whole mount stereo electron microscopy. Figs. 2 and 3 compare at different magnifications cytoskeletal organization of untreated and cytochalasin D–treated cells. The cytoskeleton of untreated BSC-1 cells is characterized by a complex three-dimensional network composed of actin filaments, intermediate
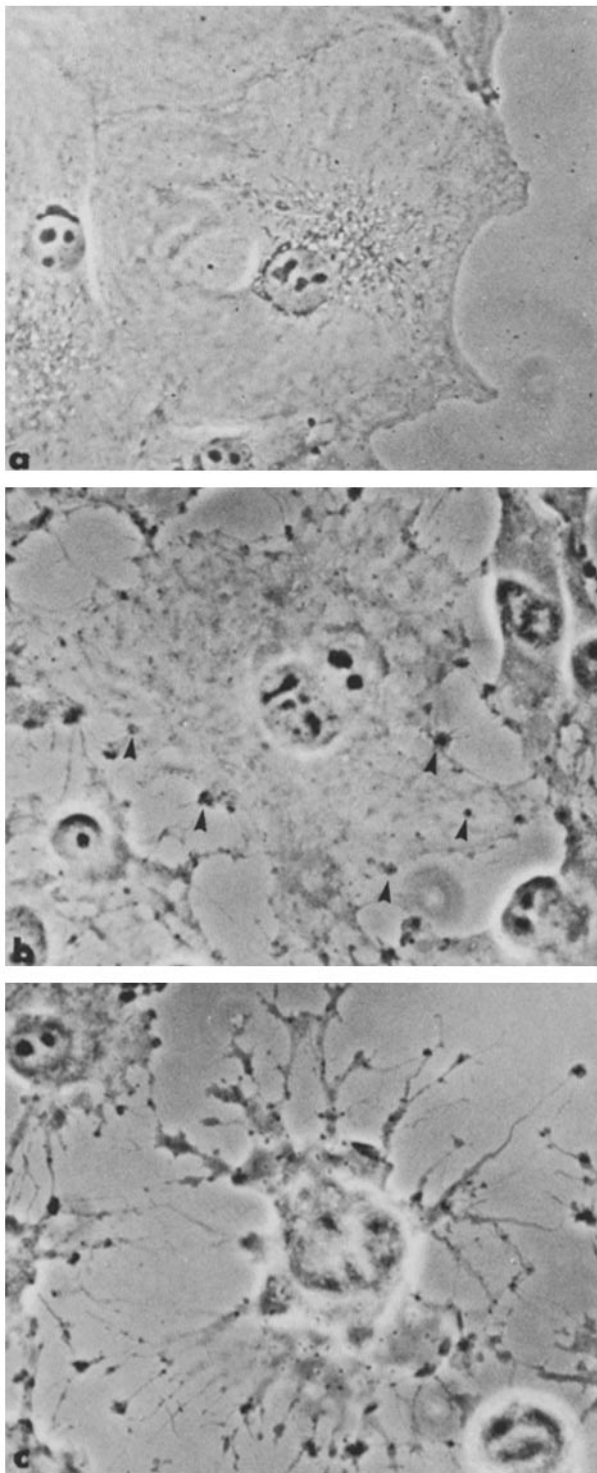
FIGURE 1 Phase-contrast light micrographs of control and cytochalasin D treated BSC-1 cells. (a) Untreated control cell. (b) 15-min treatment with 2 μg/ml cytochalasin D. Phase-dense foci have formed predominantly in the cell periphery (arrowheads). Cell retraction has just begun. (c) 45-min treatment with 2 μg/ml cytochalasin D. Cell retraction and arborization are almost complete. × 450.

filaments, 2- to 3-nm filaments, and microtubules overlying substrate-associated filament cables (31). Stereo electron microscopy of Triton-extracted BSC-1 cells shows the 2- to 3-nm filaments to act as linkers between other cytoskeletal components, including microtubules, intermediate filaments, and ac-

tin microfilaments (Fig. 4a). Although a minor component by mass (compared to, for example, actin filaments), their apparent cross-linker function makes them an important component in cytoskeletal consolidation and filament-filament interactions (31). In addition to 2- to 3-nm filaments, actin filaments are also frequently observed to form end-to-side associations with other actin filaments (Fig. 4) as well as microtubules and intermediate filaments (31). The abundance of such connections make the cytoskeleton appear as a highly interconnected continuum in which the precise organization of each of the filament types appears to depend on the integrity of the other cytoskeletal fibers. This image of network organization is markedly affected upon cytochalasin D treatment in a way which can probably best be described as "disruptive." The filamentous reticulum still shows all the components present in untreated cells; however, it is rather irregular and non-uniformly distributed throughout the cell. Some cell areas appear completely devoid of filaments, whereas in others dense network aggregates, clumps, or even compact foci of filamentous material have formed (see Figs. 2b and 3b). Labeling with heavy meromyosin-S 1 discloses actin filaments as the major filament species associated with aggregates or dense foci (Fig. 4b). Microtubules and intermediate filaments still appear intact, although their distribution has changed, too, possibly as a consequence of the reorganization of the actin filament network. Both microtubules and intermediate filaments frequently change their course in the vicinity of filament foci as if they were pulled towards the aggregate; a striking example is shown in Fig. 4b. In some cell areas, the frequency of contacts of actin filaments with microtubules, whether they are end-to-side or lateral, has decreased (Fig. 5a; see also Table I in reference 31). Some microtubules appear to be suspended in filament aggregates only at a few widely separated points, bridging huge filament-free gaps (Figs. 2b, 3b, and 5a). However, many contacts of actin filaments with other cytoskeletal fibers apparently survive cytochalasin treatment (Figs. 4a and 5b). As in untreated cells (31), actin filaments in end-to-side contact with a microtubule have their arrowheads pointing towards the site of contact. Likewise, actin-filament contacts, which are most frequent close to filament foci (Fig. 4b), are made through the pointed filament end (Figs. 4b and 5b, and Table I).

Cytochalasin D Applied to the Cytoskeleton after Triton X-100 Extraction

To test whether or not cytochalasin D directly affects cytoskeletal networks, cells were first extracted with Triton X-100, and cytoskeletons were then treated with 2 μg/ml cytochalasin D for 1-15 min. Although this treatment does not induce stress fiber disintegration or the formation of dense foci (Fig. 6a), it has a profound effect on the integrity of the filament network extending throughout the cell body. Unlike control cells, the network appears discontinuous in many places; there are cell areas which do not show a network organization at all. Many filaments appear as if they are broken, cut, or disconnected from contact points to other filaments (Fig. 6b). The number of free filament ends visible in these preparations has increased approximately threefold over control cells.

The presence of discontinuities or gaps in the filament network suggests that cytochalasin D may disrupt network organization by removing some of its component filaments. This could be because of either partial depolymerization of, e.g., actin filaments, or disconnection of network components and release into the medium. Since prolonged exposure of
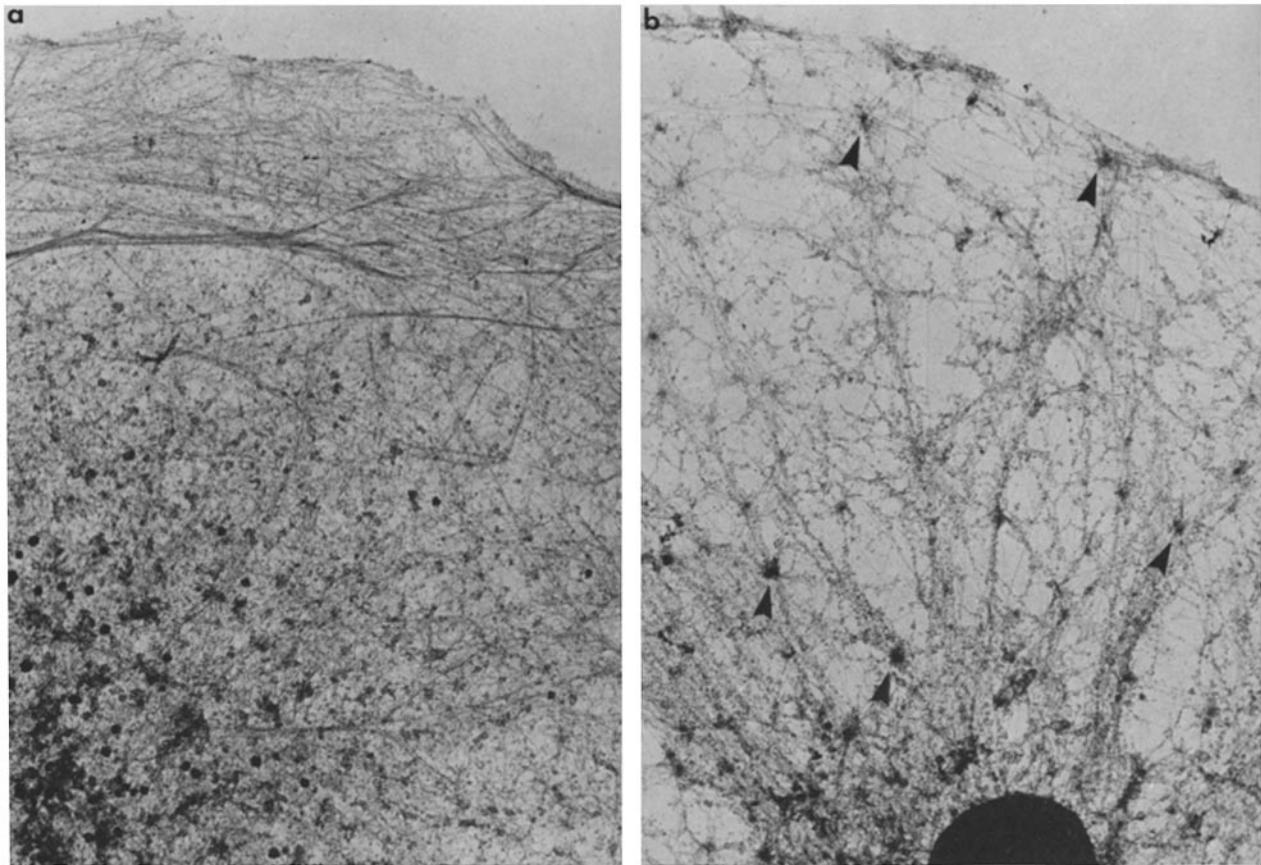
FIGURE 2 Whole-mount preparations of Triton X-100-extracted cells viewed in a high-voltage electron microscope. (a) Control cell. Note presence of filament bundles (stress fibers) and more or less uniform filament network extending throughout the cell. (b) Cell treated with 2 μg/ml cytochalasin D for 15 min. Numerous dense foci (arrowheads) have formed throughout the cytoplasm. The filamentous network is discontinuous; large areas appear to be devoid of filament network. Stress fibers are absent. × 4,500.

cytoskeletons to cytochalasin D does not increase the degree of network disruption, depolymerization seems unlikely. Therefore, the second possibility has been explored. If disconnection and/or breaking of filaments with subsequent release into the buffer occurs, it should be possible to recover released components in the supernatant. Negative staining of the supernate of cytochalasin-treated cytoskeletons reveals numerous filament fragments, 0.2–4 μm in length which, by virtue of their decoration with S1, can be shown to be F-actin (Fig. 7). Filament fragments may also be encountered in supernates of DMSO-treated control cells, but at a six to ten times lower frequency (data based on three independent experiments). Interestingly, supernates of cytochalasin-treated cytoskeletons frequently show what appear to be network fragments, i.e., short actin filaments in end-to-side contact with another actin filament (Fig. 7). If decorated with S1, arrowheads point towards the site of contact.

Increased release of actin into the supernate upon cytochalasin D treatment of cytoskeletons was confirmed by SDS PAGE (Fig. 8). Densitometry reveals an approximately sevenfold increase in the amount of a protein comigrating with rabbit skeletal muscle actin. Other polypeptides enriched in supernates of cytochalasin D-treated preparations are bands of 100,000 mol wt and a polypeptide of approximately 200,000 mol wt that does not comigrate with rabbit skeletal muscle myosin. Several other bands also show a slight increase in intensity. In general, there is an approximately two and a half

fold increase in total proteins released upon cytochalasin treatment vs. DMSO treatment, if the protein concentration in supernate derived from an equal number of cells is determined.

Metabolic Inhibitors

Brief pretreatment of cells with inhibitors of energy metabolism before cytochalasin treatment abolishes the marked changes in cell shape, dissolution of filament cables, and formation of dense filamentous aggregates (3, 15, 26). To test whether such a treatment also inhibits, or otherwise influences, cytochalasin-induced disruption of the three-dimensional cytoskeletal filament network, BSC-1 cells were pretreated with oligomycin, dinitrophenol, 2-deoxyglucose, or combinations of these inhibitors of energy metabolism. In these experiments, gross morphological changes normally occurring in cytochalasin-treated, uninhibited cells are blocked. Cytoskeletal preparations, however, reveal marked changes in filament network arrangement (Fig. 9) resembling those observed in cytochalasin-treated cytoskeletons (see Fig. 7). The filament network is disrupted in many places occasionally in such a way that "holes" of up to 1-μm diameter appear in the network. Numerous free filament ends are observed. Occasionally, zones of increased filament density, which may be interpreted as early stages of filament foci formation, are encountered in the cell periphery. Stress fibers still appear intact. Disruption of the filament network without stress fiber dissolution or pronounced
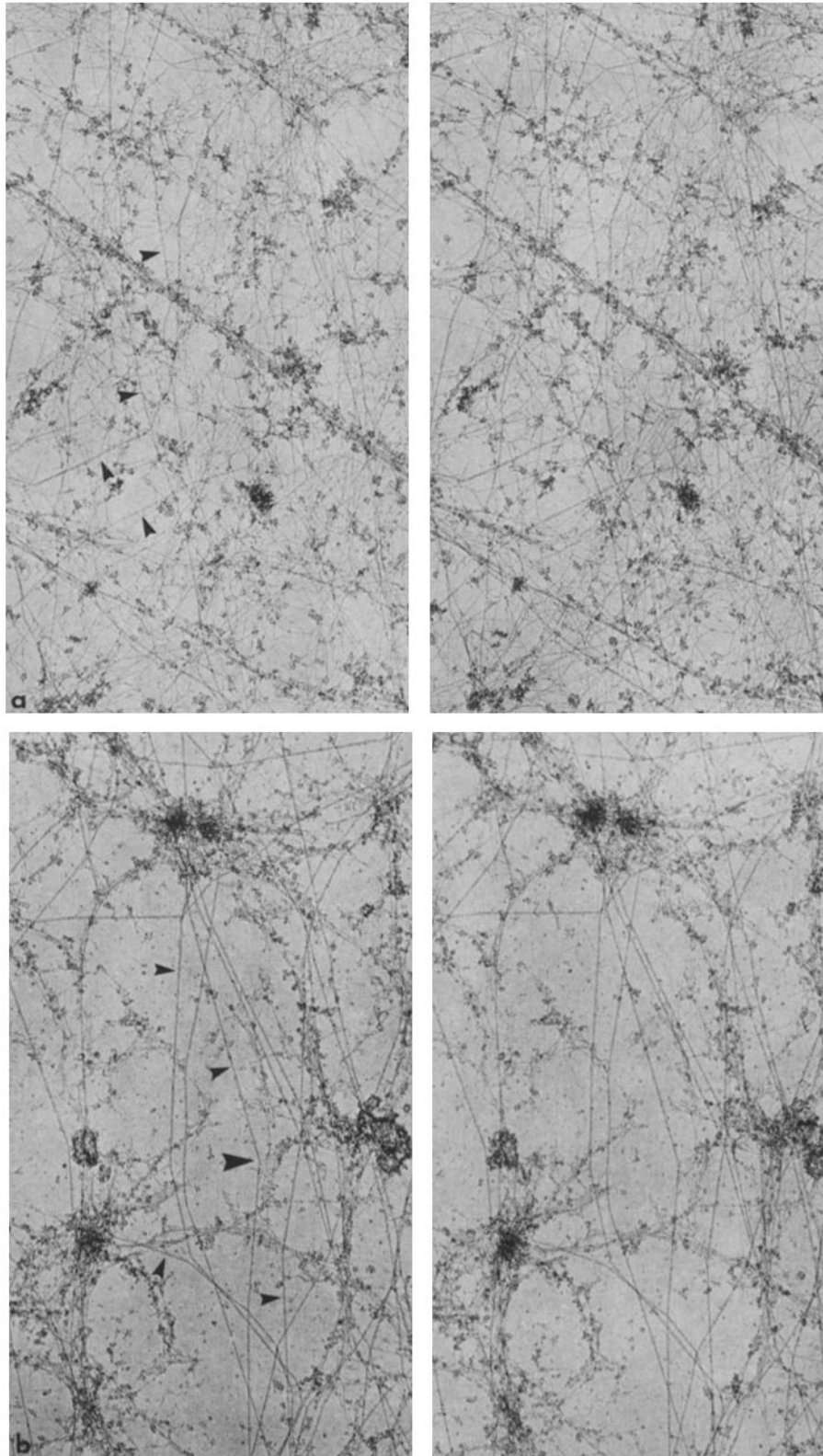
FIGURE 3 Stereo pairs of untreated and cytochalasin D–treated cytoskeletons. (a) Untreated cell. The cytoskeleton visualized after Triton X-100 extraction comprises an intricate network of filaments in which microtubules (arrowheads) are suspended. Some filament bundles are located near the substrate-side of the cell. Numerous ribosomes (dense spherical particles) are still associated with cytoskeletal filaments. (b) 15-min treatment with cytochalasin D (2 μg/ml). The three-dimensional filament network has been disrupted to the extent that microtubules (small arrowheads) bridge large filament-free cytoplasmic areas. Filamentous material has accumulated in dense foci and in patches near the substrate side of the cell. Numerous ribosomes are still associated with filaments. Large arrowhead denotes end-to-side contact of a filament with a microtubule. × 21,000.
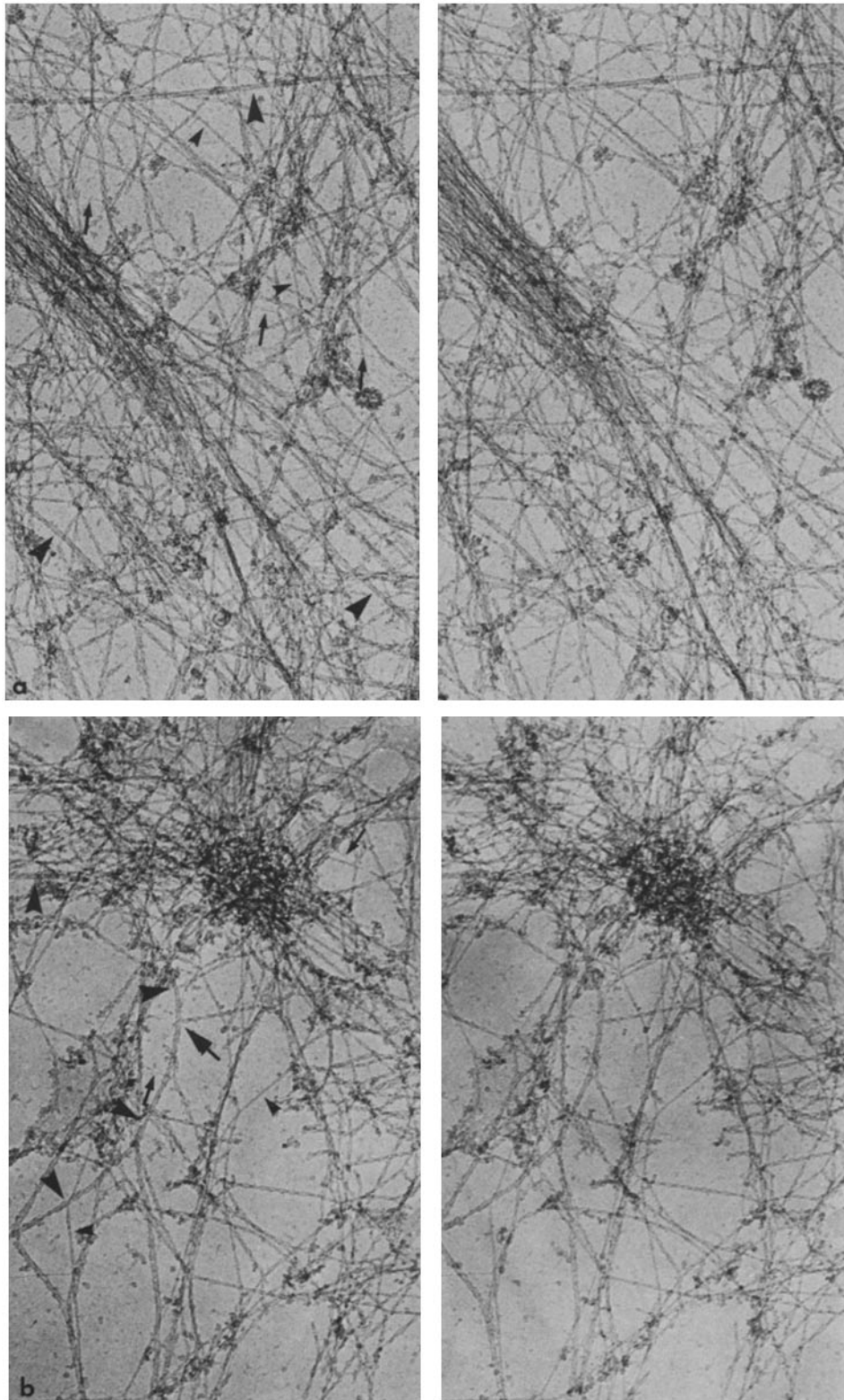
FIGURE 4 Heavy meromyosin–S1 labeling of cytoskeletons of untreated and cytochalasin D-treated cells. (a) Untreated cell. Actin filaments are labeled with periodic arrowhead patterns. Undecorated intermediate filaments (small arrowheads) can easily be distinguished from S1-labeled actin filaments. 2- to 3-nm filaments act as linkers between other cytoskeletal fibers (small arrows). Microtubules are denoted by large arrowheads. (b) Similar region of a cell treated with 2 μg/ml cytochalasin D for 15 min. All the cytoskeletal components of untreated cells are also present in cytochalasin-treated cells: microtubules (large arrowheads), intermediate filaments (small arrowheads), 2- to 3-nm filaments (small arrows), and S1-decorated actin filaments. The latter are most abundant near the filamentous aggregate, from which many filaments appear to emerge in a more or less radial fashion. The large arrow denotes an actin filament in end-to-side contact with a microtubule. Note the extreme curvature of the microtubule marked by a series of large arrowheads. × 43,000.
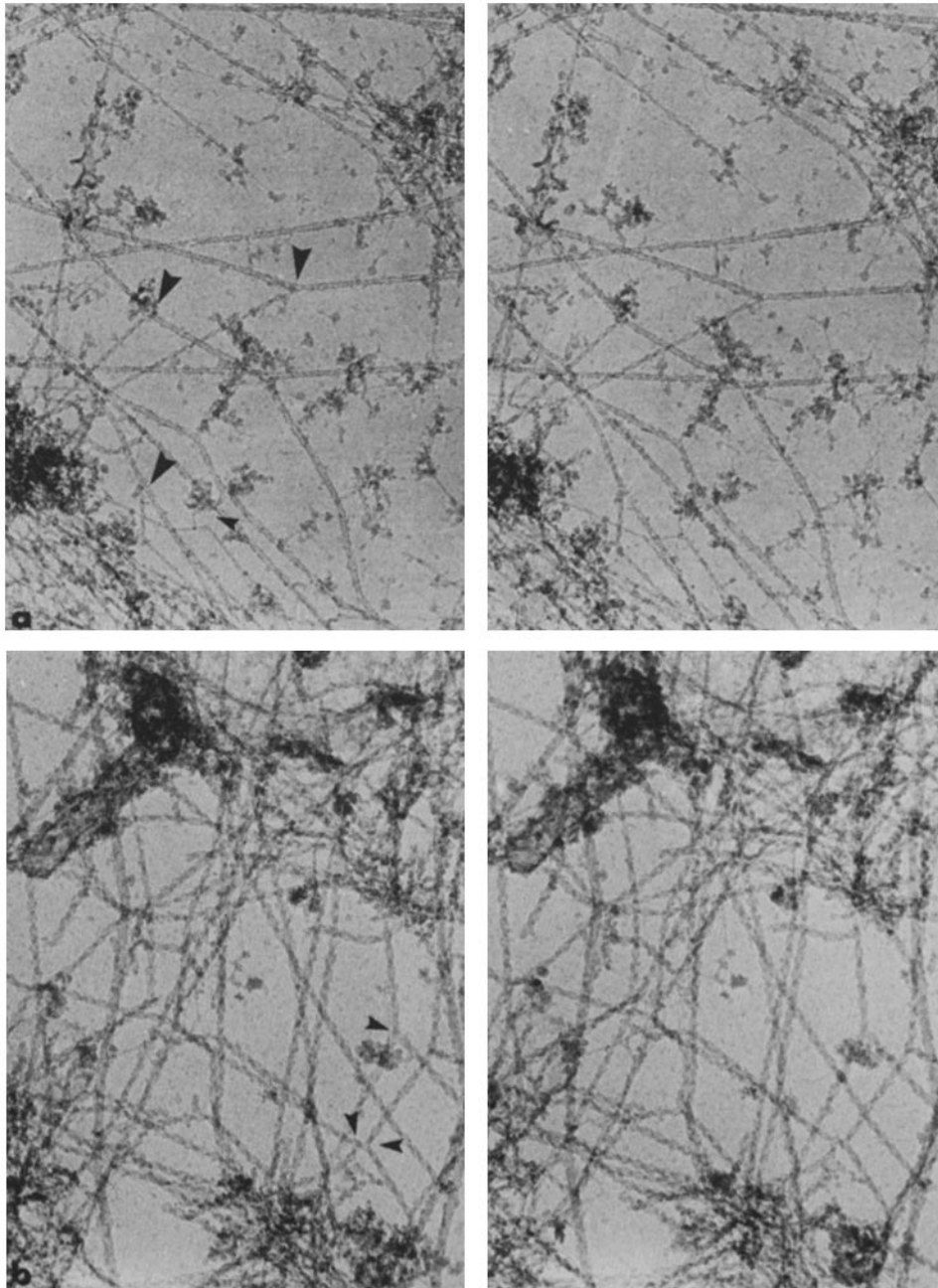
FIGURE 5 Structural interactions between cytoskeletal components in cytochalasin D-treated cells. (*a*) Actin filaments forming end-to-side contacts with microtubules (arrowheads). The pointed end of the S1-labeled filaments are in contact with the microtubule surface. Apart from these contacts, this cell area lacks a filament network. Small arrowhead denotes a 2- to 3-nm filament interconnecting a microtubule and an intermediate filament. × 54,000. (*b*) End-to-side contacts of actin filaments with other actin filaments in a "branch" type pattern (arrowheads). × 98,000.

foci formation is also observed if cells are treated with cytochalasin D for only 1–3 min (not shown).

Foci Formation: Model Experiments Using Actomyosin

The metabolic inhibitor studies suggest that formation of filament foci is an energy-requiring process that probably is the result of localized network contraction. This conclusion is supported by immunofluorescence observations showing an accumulation of actin, myosin, and tropomyosin in cytochalasin-induced foci (15, 36). To test whether isotonic contraction

of a random filament network containing contractile proteins can result in aggregate formation, networks composed of precipitated actomyosin droplets are used as a model system. Such networks bear morphological resemblance to the actin-based web extending throughout cells. Filament networks derived from rabbit skeletal muscle actomyosin approximating the size of a cell are produced as described in *Materials and Methods* (Fig. 10*a*) and exposed to 2 mM ATP for 2 min (Fig. 10*b*). Upon ATP addition, the open actomyosin network contracts, thereby tearing the net apart. Instead, numerous dense aggregates composed of tightly packed filaments have formed which often are still interconnected by small filament bundles. Con-

TABLE I

Polarity of Actin Filaments at End-to-side Contacts

	Control	2 µM cyto-chalasin D, 20 min
Pointed end contacts	38	34
Barbed end contacts	1	0
Ambiguous	11	16

50 end-to-side associations were examined under each experimental condition, and the number of pointed and barbed end contacts was determined. Since in some instances actin filament polarity can not be determined with absolute certainty, a category of ambiguous cases is added to the table. Data were collected in eight different cells from three different specimen preparations.

traction of the actomyosin network and filament aggregate formation is not inhibited by cytochalasin D (up to 5 µg/ml).

DISCUSSION

The results presented here document that the three-dimensional cellular filament network undergoes a dramatic reorganization upon cytochalasin treatment. Portions of the actin filament network are drawn into clumps or foci; as stress fibers gradually disintegrate and shorten, their constituent filaments (or filament fragments) are also aggregated into foci, as suggested by the close juxtaposition of fraying stress fibers and foci. Some cell areas may be completely cleared of a filament network, appearing "empty" in cytoskeletal preparations. Because of the association of actin filaments with other cytoskeletal components, such as microtubules and intermediate filaments, the arrangement and course of these organelles is affected as well, although they still are morphologically intact (31, 36).

On the basis of the experiments and observations reported here, it is proposed that many of the effects of cytochalasin on cell morphology may result from a combination of a direct interaction of the drug with filamentous components, and secondary cellular reactions. We have identified three conditions under which cytochalasin D treatment will cause a severe disruption of the three-dimensional filament network without causing alterations in overall cell shape, disintegration of stress fibers, or formation of filament foci: (a) short (1–3 min) treatment of cells with cytochalasin D; (b) combined treatment with metabolic inhibitors and cytochalasin D for longer time periods (20–30 min); and (c) exposure of Triton X-100-extracted cytoskeletons to cytochalasin D.

From this it may be concluded that cytochalasin's most direct action results in an interference with cellular actin-based filament networks that leads to the disruption of network organization. Foci formation and stress fiber disintegration are secondary, energy-requiring processes that possibly proceed without direct involvement of cytochalasin, although they require its continuous presence. In the presence of inhibitors of
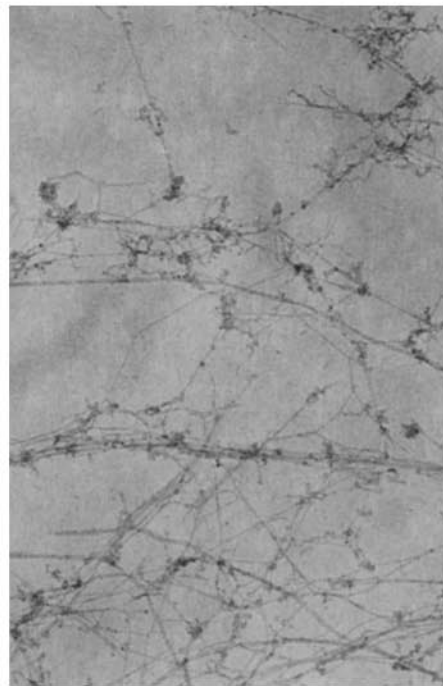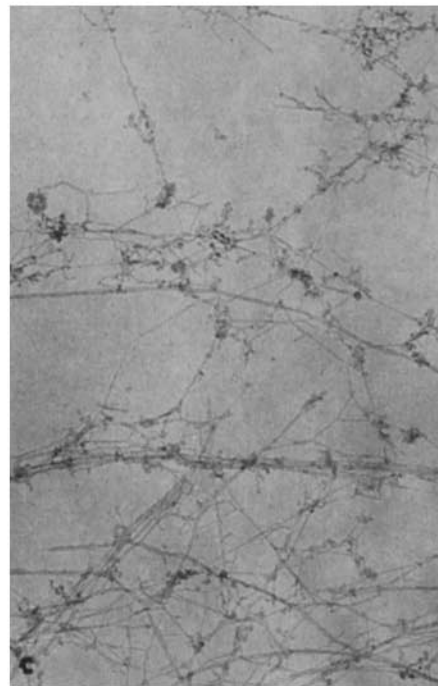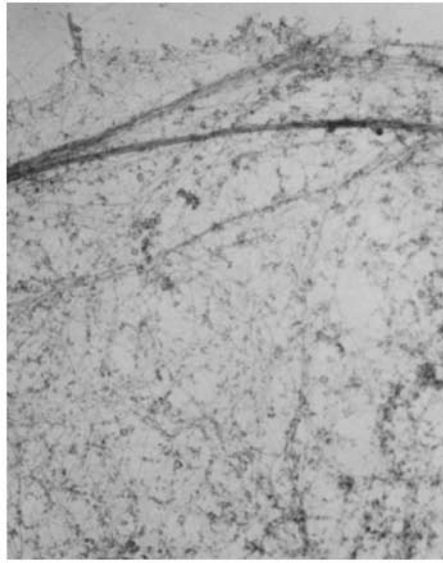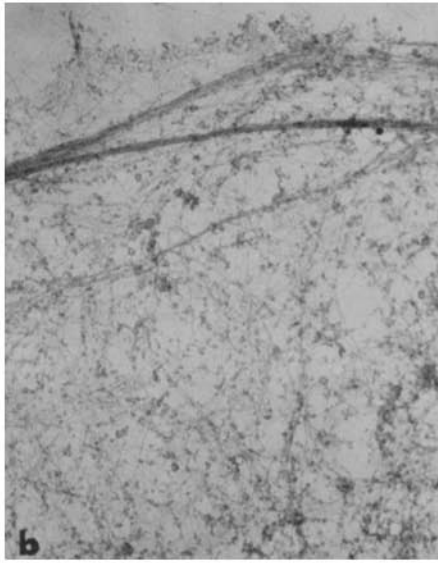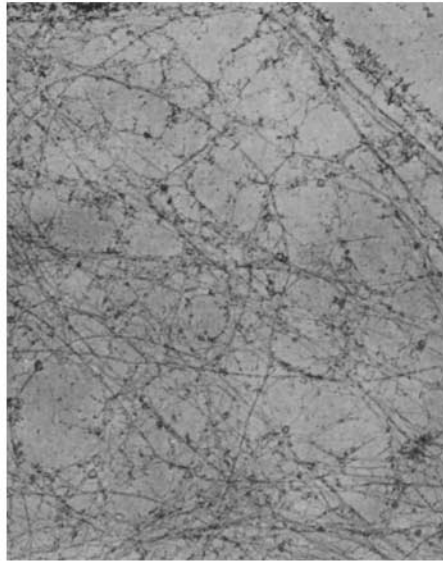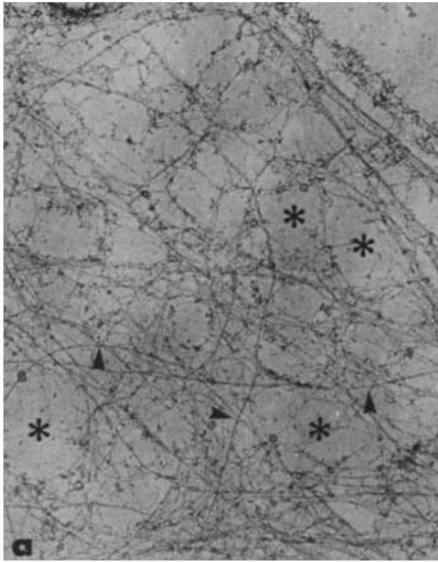
energy metabolism, cells remain in a state of network disruption without proceeding through energy-dependent stages of filament reorganization.

Site of Action of Cytochalasin

The observation of disruption of cytoplasmic filament networks by cytochalasin is consistent with in vitro analyses demonstrating interference with the formation of, and dissolution of formed, actin gels (18, 19, 24, 38). Two alternatives have been proposed to explain these in vitro effects: (a) cytochalasin may bind to the barbed end of actin filaments, thereby interfering with filament-filament interactions which contribute to network formation (22, 24); and (b) cytochalasin interferes with gel formation by severing or breaking actin filaments at limited sites (19, 32).

There is strong evidence that the preferential binding site for cytochalasin is the "barbed" end of the actin filament, and the hypothesis that it is the binding to this site that also is responsible for the drug's interference with network formation is a very attractive one. However, the possiblity of a "severing" action of cytochalasin should not be easily dismissed and should be taken into serious consideration. In fact, the observations presented here provide some evidence in favor of this alternative. Most, if not all, actin filaments in end-to-side contact with other fibers (F-actin, intermediate filaments, and microtubules) have their arrowheads resulting from S1-decoration pointing towards the contact side. It is questionable, therefore, that actin filaments in contact with other cytoskeletal fibers through their barbed end are a significant factor in network consolidation (at least in the cell type used here), if they exist at all. Binding to the barbed filament end is, however, the mechansim proposed by MacLean-Fletcher and Pollard (24) to result in inhibition of actin filament-filament interactions and network disruption. Moreover, it can be demonstrated that many of the pointed end-contacts survive cytochalasin treatment (Figs. 4b and 5), although network organization is severely affected. The interpretation advanced here is that actin filament pieces, or fragments of network structures, are "cut" out of the highly interconnected cytoskeletal network. Such fragments can be recovered in supernates of in vitro preparations. The mechanism by which cytochalasin might effect scission of actin filaments remains obscure, but an action somewhat analogous to that of the Ca^{++}-dependent actin modulating proteins, villin, fragmin, and gelsolin (for an overview, see reference 30) could be envisaged. An alternative possibility, although a less likely one in the light of the experiments with cytoskeletons (Fig. 6), is that cytochalasins block the annealing of spontaneously breaking filaments (25). Both interpretations do not exclude the possibility that network organization is also disrupted at some sites of filament-filament contacts, but such an action seems to play a minor role. Unfortunately, it is not possible to test Hartwig and Stossel's proposal (19) that cytochalasin decreases the mean actin filament length, since it is virtually impossible to identify both endpoints of an actin

FIGURE 6   Effect of cytochalasin D on the cytoskeleton after extraction. Cells were treated with 0.1% Triton X-100 in PHEM buffer for 2 min, washed with buffer, and then treated with 2 µg/ml cytochalasin D for 5 min. (a) Overview of part of a BSC-1 cell. Filament bundles and microtubules (arrowheads) are still intact, but the uniform three-dimensional network has been disrupted to the extent that numerous open areas or "holes" (asterisks) are apparent. X 12,000. (b) control cell treated with 0.2% DMSO only. The three-dimensional filament network is intact. X 12,000. (c) Higher magnification of a cell treated as in a. The filament network is disrupted and numerous free filament ends are visible. X 34,000.

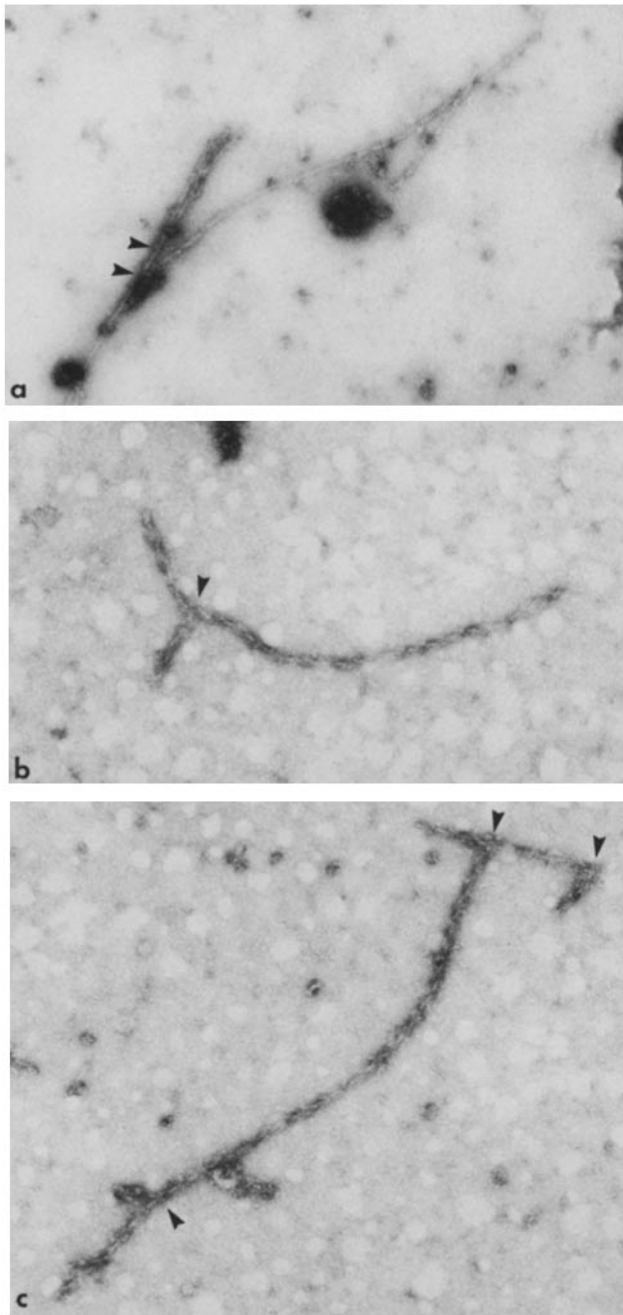treatment may be subdivided into three events which, in the order described here, also represent a temporal sequence.

(a) Within seconds or minutes of addition to cultured cells, cytochalasin directly interacts with actin-based cytoskeletal networks in a way which results in their "disruption." This process, which is independent of metabolic energy, is the cellular correlate of the recently described inhibitory action of cytochalasins on gelation of actin alone, or actin and cross-linking proteins (17, 19, 24). The mechanism by which cytochalasin exerts this effect seems to involve "breaking" of actin filaments into small fragments. Interference with actin filament-filament interactions by binding to the barbed end of the filament, as suggested by MacLean-Fletcher and Pollard (24), seems unlikely on two grounds: (i) end-to-end contacts of actin filaments with other cytoskeletal fibers appear to be exclusively made through the pointed end of the filament; and (ii) numerous such contacts can still be observed >15 min after exposure to the drug, long after network disruption has occurred. Cytochalasin may, however, bind to ends of filament fragments produced by breaking and may inhibit their spontaneous reelongation. This action, which would be consistent with its inhibitory effect on actin polymerization in vitro, may play an important role in the maintenance of the state of filament disruption in the presence of the drug.

(b) Minutes after the addition of cytochalasin to cells, formation of dense foci begins. They include not only actin filaments, often in a radial arrangement, but also myosin and tropomyosin (15, 16, 36) and probably are the result of disorganized and uncontrolled cytoplasmic contractions. These contractions accumulate actin filament and network fragments in localized cell areas and concomitantly initiate, as a result of the "clearing" of large cell areas from an ordered three-dimen-
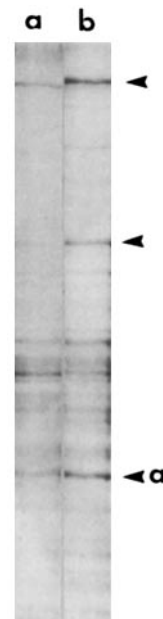
FIGURE 7 Negatively stained supernate of cell preparations treated as described in the legend to Fig. 6. Network fragments of filaments end-to-side contact (arrowheads). (a) Undecorated; (b and c) decorated with S1. In b and c the pointed filament end can be seen to be in contact with another filament. X 92,000.

filament in cytoskeletal preparations. It is clear, however, that cytochalasin treatment of cytoskeletons increases the number of free filament ends substantially, an observation consistent with a proposed increase in filament number by scission. Whether the proposed mechanism of cytochalasin D action on cellular actin networks is due to an effect on either the low or the high affinity binding sites on F-actin or affects both classes of binding sites cannot be said with certainty.

Cytochalasin-induced Cellular Reponses: A Model

The structural changes ensuing in cells upon cytochalasin

FIGURE 8 PAGE of proteins released from cytoskeletons by DMSO or cytochalasin D treatment. 10^6 BSC-1 cells grown in plastic petri dishes were extracted with 0.1% Triton X-100 in PHEM buffer, washed twice with buffer, and then treated with 0.2% DMSO or 2 μg/ml cytochalasin D for 5 min in buffer. The supernates were removed, lyophilized, and run on 7.5% slab gels containing SDS. (a) Supernate of a DMSO-treated culture. (b) Supernatant of a cytochalasin D-treated culture. Polypeptides enriched in gel b are indicated by arrowheads. a, actin.
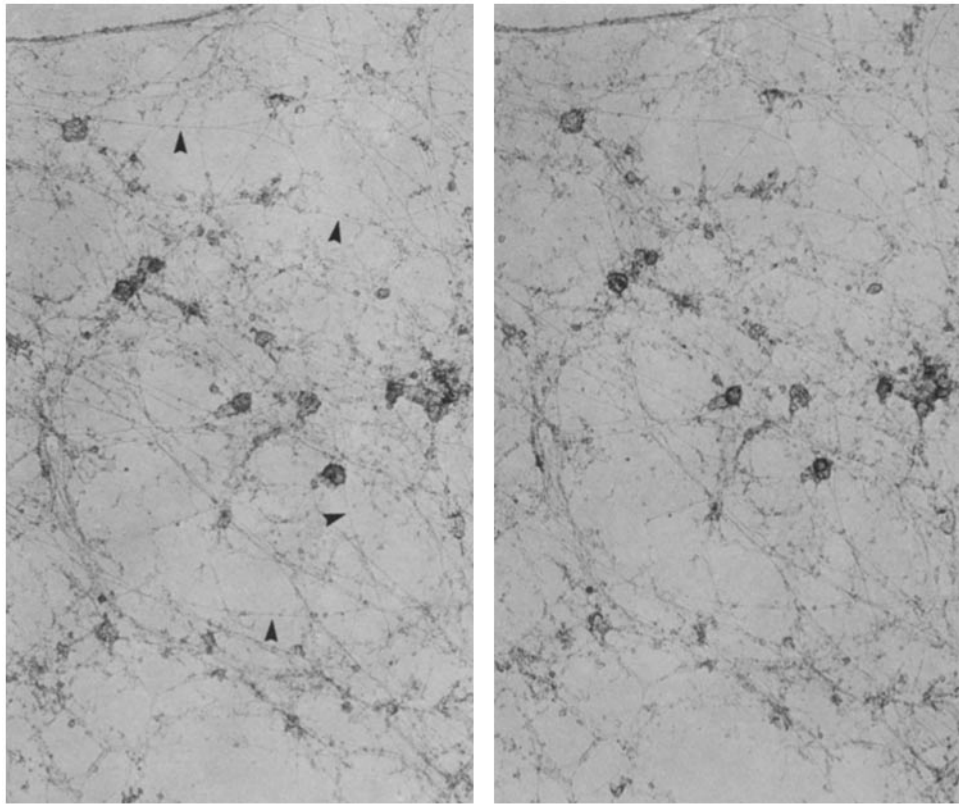
Figure 9   Part of the cytoskeleton of a cell treated with 2-deoxyglucose and dinitrophenol for 30 min, followed by a 20-min treatment with 2 µg/ml cytochalasin D in the continued presence of the metabolic inhibitors. The three-dimensional filament network extending throughout the cell body is severely disrupted. Arrowheads denote microtubules. × 65,000.

sional filamentous network, cell retraction and arborization. "Unremittent contraction" has already been proposed by Godman and Miranda (14) and more recently by Godman et al. (15) to be responsible for some aspects of the redistribution of cytoplasmic contractile proteins upon cytochalasin treatment.

(c) Disintegration of stress fibers is the latest of the morphological events observed after cytochalasin treatment, and also the least understood. Since the cellular pool of actin in the polymerized state is not significantly decreased upon cytochalasin treatment (34), depolymerization does not seem to be involved. On the other hand, stress fiber components do most probably become part of the actin-containing foci. According to Godman et al. (15), stress fiber disintegration involves their shortening because of contraction and direct incorporation of the stress fiber material into foci. However, treatment of cultured cells with metabolic inhibitors may also lead to slow shortening and disappearance of stress fibers (3), although in these cells contraction is inhibited. An alternative interpretation advanced here is that there is a turnover of F-actin between the "network" and the "cable" state. In fact, actin filaments emerging from cables and becoming part of the open three-dimensional network are frequently observed (not shown). In the presence of cytochalasin, actin filaments may still be able to leave stress fibers; if they subsequently become redistributed into filament foci, reentry of actin into cables would be shut down. As a consequence, filament bundles gradually "dissolve."

Although cytochalasins exert a wide variety of most dramatic responses in many cell types, it appears that many of its effects are the consequence of an immediate and energy-independent action on the organization of cytoskeletal networks. The experiments described here emphasize the importance of an intact three-dimensional filamentous network with its numerous interactions and cross-connections for the maintenance of cell integrity. Since the pattern of interactions between cytoskeletal components may differ from one cell type to another and may even vary among the cells in the same culture, their responses to cytochalasin may show some variations as well. Such variations in the response to cytochalasin of different cell types are well illustrated in the two papers by Godman et al. (15, 16). If this argument can be turned around, then variations in the response to cytochalasin may be taken as an indicator of substantial differences in cytoskeletal network organization and filament interaction.
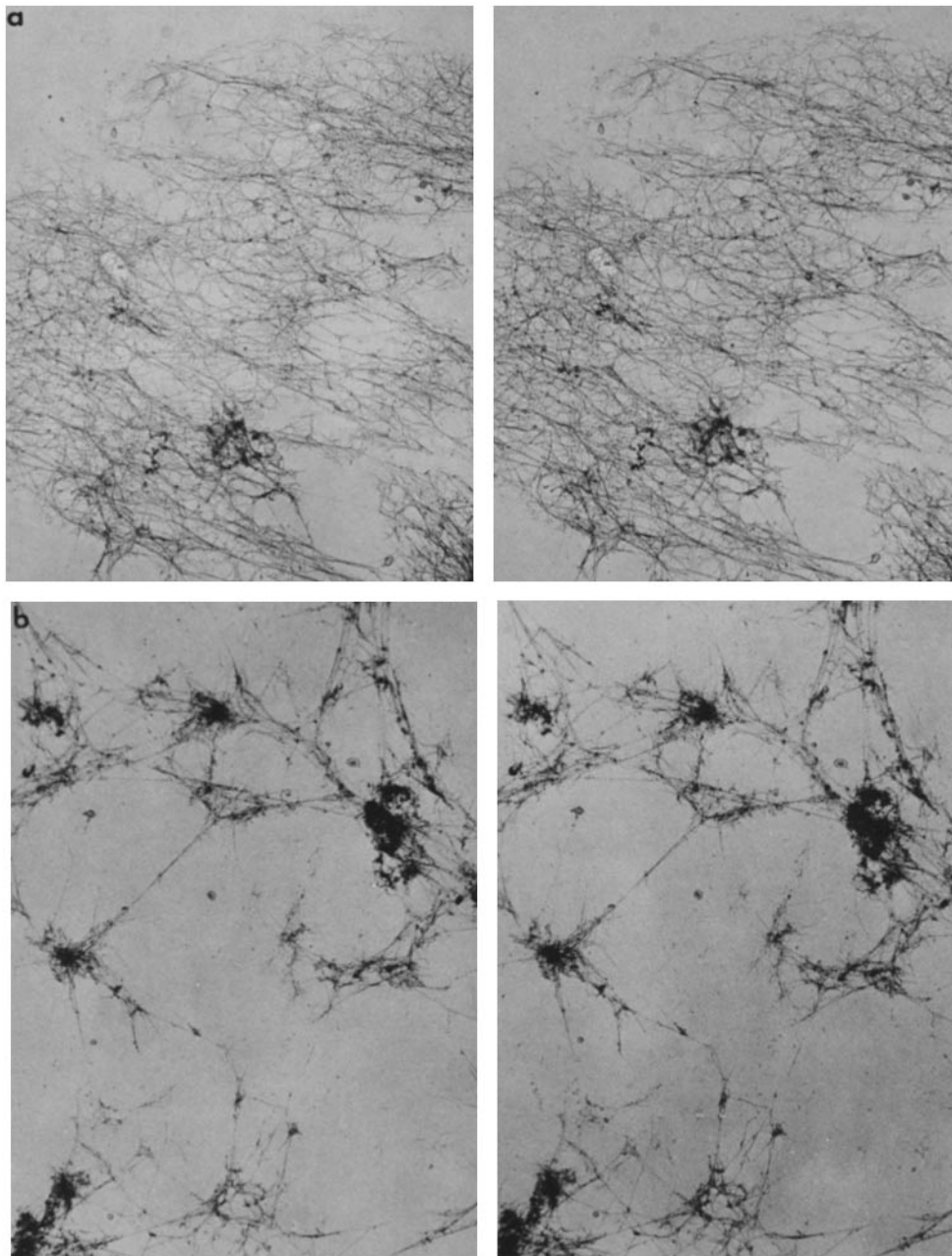
FIGURE 10 Actomyosin droplets precipitated onto the Formvar film of an electron microscopic grid. (a) In the absence of added ATP. The precipitated actomyosin forms a three-dimensional filamentous network. (b) Actomyosin droplet prepared as in a, but subsequently treated with 2 mM ATP for 2 min. The actomyosin network has contracted. Upon contraction, network organization has been disrupted, and numerous dense filamentous foci have formed. × 6,000.

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