ACTION OF CYTOCHALASIN D ON CELLS OF ESTABLISHED LINES

II. Cortex and Microfilaments

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ABSTRACT

Cells in culture exposed to cytochalasin D (CD) rapidly undergo a long-sustained tonic contraction. Coincident with this contracture the thin microfilaments of the cortex become compacted into feltlike masses. The ravelled filaments of these masses remain actinlike and bind heavy meromyosin; they are not disrupted or disaggregated, but rather, appear to represent a contracted state of the microfilament apparatus of the cell cortex. On continued exposure to CD, 'myoid' bundles, containing thick, dense filaments, and larger fusiform or ribbonlike, putatively myosinoid, aggregates may appear.

These appearances are interpreted as consequences of a state of hypercontraction without relaxation induced by CD. They do not occur in CD-treated cells prevented from contracting by inhibitors of energy metabolism, and are readily reversible on withdrawal of CD. Extensive ordered arrays of thin microfilaments develop in cells which are reextending during early recovery.

INTRODUCTION

In a preceding communication, we have described some of the earliest consequences of the action of cytochalasin D (CD) on cells in culture (36). The most remarkable of these is a state of contracture, or long-sustained contraction. Like other visible phenomena with which it is accompanied (viz: inhibition of locomotion and movement, protrusion of endoplasm and nucleus), contracture occurs at low to moderate concentrations of CD; it is dose dependent, requires energy, and is readily reversible. Since the intracellular tension developed during contracture would appear to be a principal cause of such other striking effects of CD as zeiosis and nuclear protrusion, the induction of cell contraction by CD may be a phenomenon of central importance in understanding how the cytochalasins act. We have considered, in the light of current concepts of cellular contractility, some of the possible ways in which CD could effect cell contraction (36).

To help define the events occurring in the contractile apparatus of the cortical cytoplasm during CD-induced contracture and after withdrawal of CD, changes visible in the various microfilaments of the cytoplasm at intervals after exposure to this agent were examined. The appear-

ances depicted in this communication suggest that the remarkable changes effected by CD in the motor apparatus of the cell cortex are a visible concomitant of the hypercontracted state, and appear not to result from structural disruption of microfilaments.

MATERIALS AND METHODS

Monolayer cultures of HeLa, HEp2, L, Vero, MDBK, and PR-105 cells grown in monolayer were treated with medium containing cytochalasin D (CD) or DMSO (controls) and prepared for transmission electron microscopy as described previously (36).

Selected cultures of HeLa and Vero cells were pretreated for 5 min with the inhibitor of energy metabolism, 2,4-dinitrophenol (DNP; 5×10^{-3} M) or the nonmetabolizable 2-deoxyglucose (DOG; 1×10^{-2} M) (Sigma Chemical Co., St. Louis, Mo.) in glucose-free growth medium, supplemented with dialyzed newborn calf serum (Grand Island Biological Co., Grand Island, N. Y.). Cultures were then exposed for 15 min to CD (0.25 μg/ml, or 1 μg/ml) or DMSO (0.1%) in inhibitor-containing medium and fixed for electron microscopy. To identify actin or actinoid components, HeLa cells, grown in 30 ml Falcon flasks (Falcon Plastics, Div. Becton-Dickinson Laboratories, Inc., Los Angeles, Calif.), were glycerinated for 24 h, then treated with 1.5 mg/ml heavy meromyosin (HMM)[1] for 12 h according to the method described by Ishikawa et al. (26), and prepared as usual for electron microscopy.

RESULTS

Microfilaments of the Cortical Cytoplasm

The chief structural component of the ectoplasm of these cells is the system of thin microfilaments of 4.5–7.0 nm (Figs. 1–3). In the cortex of the basal, or adherent aspect of the cell, the microfilaments are usually in closely spaced parallel arrays (Figs. 2, 3). In the aggregate, these arrayed filaments constitute a "sheath." The arrays may exhibit patches of increased density at irregular intervals and at some foci where they abut on the inner aspect of the plasma membrane. Arrayed microfilaments are also conspicuous in the ectoplasm of the narrower trailing ends of moving cells, especially at the sides, where the sheath sometimes appears even thicker than that of the basal sole. In the interstices where the sheath is sparser, some loose unorganized meshworks of microfilaments can be discerned.

The 4.5 to 7-nm microfilaments of the ectoplasm of the free surface form connected meshes or nets somehow attached to the inner face of the plasma membrane (Fig. 1). These meshworks are more or less compact: in the less compact regions, especially as seen in tangential sections of inner membrane, the reticulum formed by the short lengths of filament may appear as a net of polygons like those depicted by Yamada et al. (63). Small bundles of parallel microfilaments of the same thickness may course through these networks. Filaments of the meshworks and some of those that make up the bundles are continuous. Such filament arrays are relatively few and inconspicuous in cells of established lines; in contrast, in fibroblasts, such as those of the PR-105 strain, the WI38 strain or those in primary culture, they constitute the prominent 'stress-fibers' well depicted by Buckley and Porter (10). Arrays are sometimes discernible in microvilli or the smaller microspikes, and are usual in the longer microextensions.

These microfilament systems of the cortex are apparently most exiguous in the cells of the Vero line; they are best developed in MDBK cells. In monolayers of HeLa, HEp2, and L cells the abundance and organization of microfilaments is intermediate between these.

The cell cortex evidently constitutes an organized barrier between the plasma membrane and the endoplasm that normally prevents the main endoplasmic contents from approaching the cell membrane, except for the occasional isolated segments of endoplasmic reticulum some of which may almost reach its inner surface. Both smooth and coated ("basketed") vesicles in various stages of formation or discharge, however, may be numerous at the cell membrane of the free surface.

The Cortex Early after Exposure to CD

Cytochalasin D begins to act almost immediately: in HeLa, by 2–3 min definite retraction, withdrawal of microvilli, and sometimes zeiosis has occurred (36). By 4 min after 0.25 μg/ml CD well-marked changes in the surface and the cortex are evident in the electron microscope (Fig. 4 *a–b*). A compact reticulum of microfilaments in a discontinuous layer of from 0.15 to 0.4 μm (i.e. an

[1] HMM prepared from rabbit skeletal muscle myosin was kindly supplied by Dr. Sol Berl, Columbia University, New York. The stock solution (4 mg/ml) was stored in 50% glycerol at −20°C.

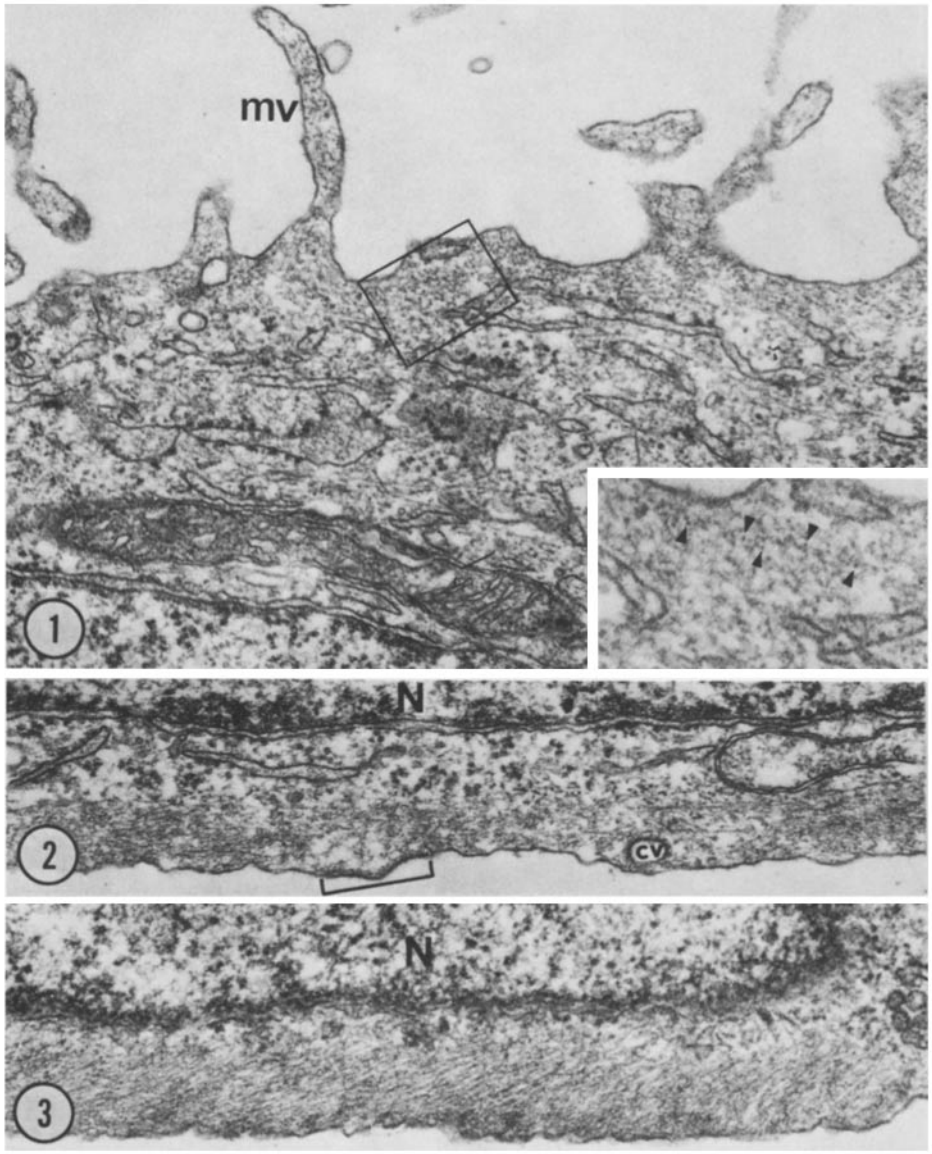


FIGURE 1 Sector of the free side of a HeLa cell in monolayer in 0.02% DMSO medium (control). A meshwork of thin (4.5–7.0 nm) microfilaments forming a continuous layer about 0.12 μm thick occupies the superficial cortex subjacent to plasma membrane, into the cytoplasmic side of which the filaments attach. Some are associated with 7 to 8-nm densities such as those marked with arrowheads in the insert. The filament web extends into the microvilli (*mv*). Endoplasmic contents between the microfilament mesh and the nucleus (*N*) are excluded from the network and prevented from impinging on the plasma membrane. × 40,800. Inset, × 83,600.

FIGURE 2 Sector of the basal side (sole) of a HeLa cell in monolayer in 0.02% DMSO medium (control). Thin microfilaments of the cortex are arrayed in parallel, longitudinal bundles, or laminae. Dispersed microfilaments of a loose mesh are also evident. The nucleus (*N*) is at the top. A coated vesicle is marked *cv*, and a small patch of plasma membrane, the cytoplasmic face of which is coated, the putative site of vagination, is indicated in bracket. × 40,500.

FIGURE 3 Basal aspect of an MDBK cell in monolayer showing closely stacked obliquely oriented parallel arrays of microfilaments constituting a thick cortical sheath, in this instance, of about 0.25 μm. × 45,000.

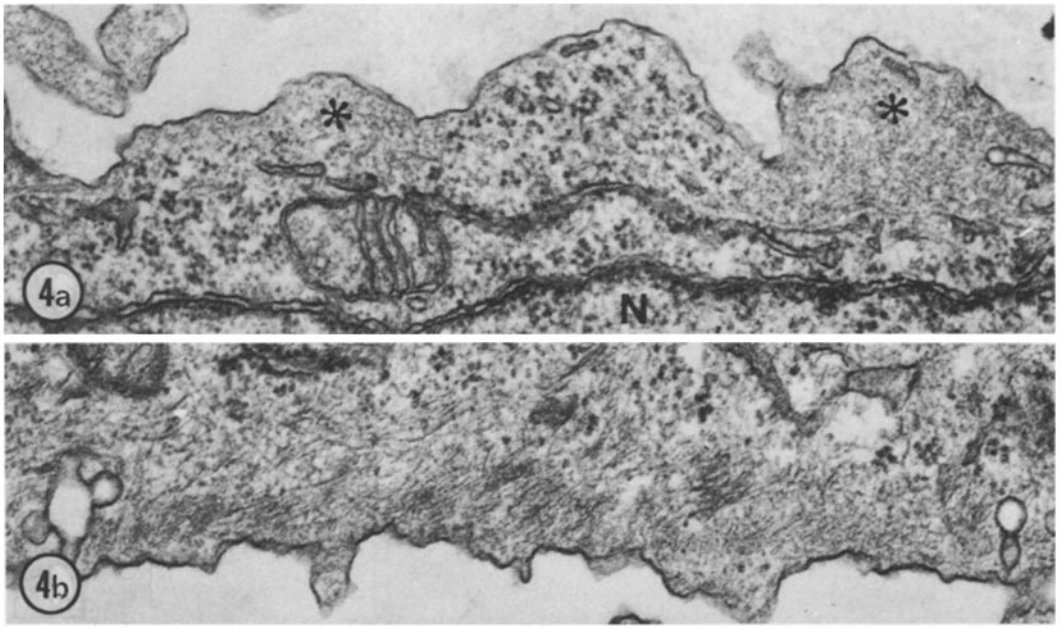


FIGURE 4 *a* Free side of a HeLa cell in monolayer after 4 min of exposure to 0.25 μg/ml of CD. The microfilaments are mostly gathered into thicker, more compact masses or pads (asterisk). The filament meshwork has become discontinuous; between the two filament masses a plug of endoplasm appears to abut against the plasma membrane and to burgeon out at the surface. This is an early stage in the formation of a zeiotic protrusion. Microvilli have mostly disappeared. × 47,000.

FIGURE 4 *b* Basal (attachment) side of a HeLa cell in monolayer after 4.5 min of exposure to 0.25 μg/ml CD. Most arrays of the sheath of microfilaments appear to be intact; small patchy arrays with denser filaments or filament segments are evident. × 48,000.

average of about 1.5–2 times the normal thickness) lies subjacent to the plasma membrane of the free surface most prominent at the forward edge. The gaps in this cortical feltwork are occupied by endoplasm, which at these loci comes to lie directly against the inner aspect of the plasma membrane; these plugs of endoplasm then burgeon beyond the surface as short blunt protuberances. These are the early or inicipient zeiotic protrusions (Fig. 4 *a*). The microvilli are reduced in number and are shortened and distorted. Some arrays of the basal sheath remain intact, but part of this sheath appears to have been replaced by loose filament meshworks (Fig. 4 *b*). A few bundles of thin filaments are also still observable in the cortex of the free side of the cell (Fig. 6).

Most of the vesicles bounded by smooth membrane disappear from the cortex and subplasmalemma very rapidly after application of CD. At 4 min their diminution is marked, and they are virtually absent by 1 h. In contrast, the coated vesicles and their precursor formations in the plasma membrane are not affected in number or appearance, even after prolonged exposure to CD.

These changes are progressive during the ensuing 1–3 h of exposure to moderate concentrations of CD (e.g. 0.25–0.5 μg/ml in HeLa; 0.5–2.0 μg/ml in MDBK). Concomitant with the progression of contracture and zeiosis, the wooly skeins of compacted microfilaments in the cortex of the unattached side of the cell accumulate into thicker masses or pads subjacent to plasma membrane (Fig. 6 *a–b*). Peripheral filament masses sometimes attain dimensions of as much as 2 μm, but most of the feltlike layer at the free surface of HeLa cells has an average thickness of about 0.7 μm (0.35–0.96 μm). Larger accumulations of this compact meshwork are prominent at the bases of the zeiotic protrusions, and extend part of the way up into their stems (Figs. 5, 7). In HeLa, after grouping of the zeiotic knobs at the apex, some 60 to 90 min after application of CD (34), this thick layer of massed filaments at the free surface under the clustered knobs has become more continuous,

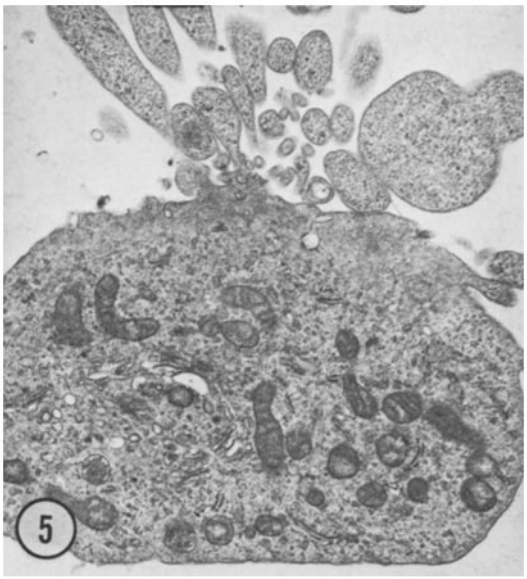


FIGURE 5 Transverse section of a contracted HeLa cell after 5 h in CD (0.25 μg/ml). The zeiotic processes have migrated centripetally to form a bouquet at the apex. The felt of compacted microfilaments is gathered into a thick pad in the apical cortex and extends into the bases of the zeiotic stalks. Elsewhere, at the sides and base of the cell the cortical microfilament net (ectoplasm) is either very thin or inapparent. × 14,800.

with few of the gaps so evident early after application of the CD (Figs. 4, 5). Although occasional thin bundles of 4.5 to 7-nm filaments in parallel arrays remain intact in the cortex, especially at the underside of the cell, most of the basal cortex like that of the free side, is also eventually occupied by filamentous felt.

Similar events are observable in cells in suspension, such as HeLa-S3, during exposure to CD. Suspended cells lack structures corresponding to a shealth of arrayed microfilaments, and their submembrane filament meshwork is normally very narrow.

Microfilament Masses

By 5 h of exposure to CD, large compact pads of the microfilamentous felt occupy the cortex, and may also extend inward to the endoplasmic region (Fig. 7). The interconnected masses may be as much as 2–3 (or more) μm thick. In thin section, they are seen to consist of kinked, branched, or connected segments presumably of randomly ravelled fibrils, about 4–8 nm in diameter (Figs. 7, 15). The interstices between the fibril segments are occupied by an extremely fine thready matrix of low density. Only rare segments of endoplasmic reticulum and some occasional microtubules are trapped within the filament feltwork; other structures are excluded. Small patches of much greater density often appear within the main mass of felt. In their looser, and possibly earlier state of development, these appear to be composed of dense, thick, closely spaced filament-segments (Fig. 7). They are sometimes seen soon after formation of the wooly bodies, but after 3–5 h of exposure to CD, more numerous, homogeneous dense bodies will have accumulated within the skeins (Figs. 7, 19). These bodies are remarkably similar to the developing Z-band regions of differentiating muscle (16, 23, 27) and to dense patches of leiomyocytes. Their number and size appear to increase with time in CD (Fig. 19).

That these early events entail displacements and redistribution of the existing filament mesh, is suggested by the rapidity of the accumulation of filamentous material in discontinuous masses (Fig. 4 *a–b*), and their later movement into the subcortex (Fig. 16) and toward the sites where zeiotic knobs congregate (Figs. 5, 7). But, in addition, the amount of microfilamentous material accumulated as a compact felt also appears to increase, at least relative to cytoplasmic volume, especially after longer exposure to CD or at higher doses (Fig. 16). However, without morphometric comparisons, we cannot definitely ascertain whether CD induces an increase in the total amount of filamentous material.

The accumulation of the filament masses is dependent on, or in any case related to the development of contracture. When CD-induced contraction and zeiosis of HeLa are prevented by treatment with either 1×10^{-2} M deoxyglucose or 5×10^{-3} M dinitrophenol for 5–10 min before and during a 15-min exposure to 0.20 μg/ml of CD (36), the cell cortex and surface are indistinguishable from untreated normal control cells. Colcemid, in concentrations sufficient to cause metaphase-arrest and the disappearance of most microtubules (i.e. 0.1–0.6 μg/ml), is without effect on the action of CD and on the visible changes that overtake the microfilament system.

Binding of Heavy Meromyosin

Further insight into the effect of CD on cortical microfilaments can be gained from an examination

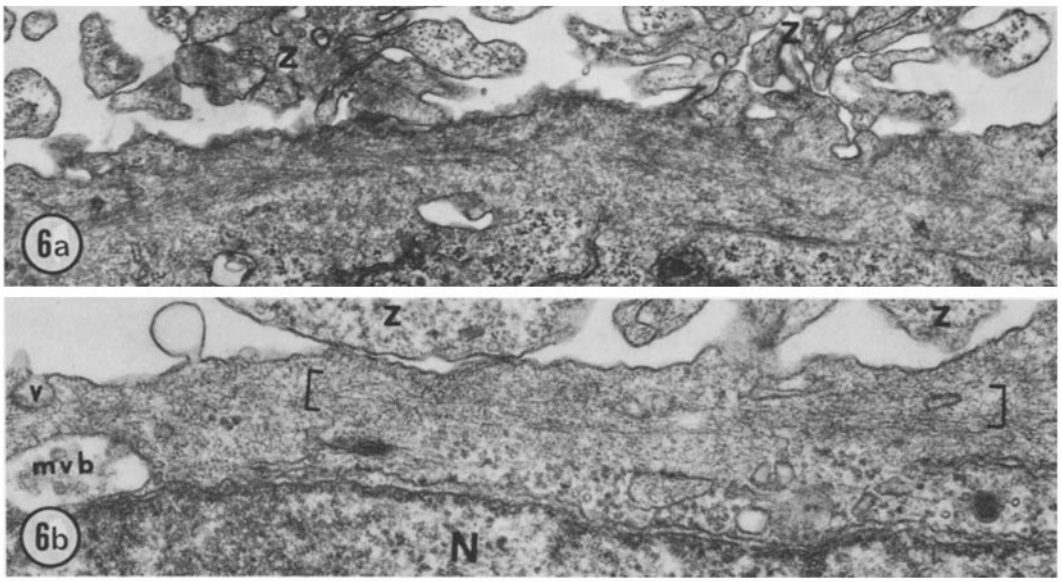


FIGURE 6 *a* Top side of a HeLa cell which has been pretreated with Colcemid (0.6 μg/ml) for 6 h and subsequently exposed to medium containing 0.6 μg/ml Colcemid plus 0.25 μg/ml CD for 1 h. Fascicles of thin microfilaments course through the cortex which is otherwise occupied by a thick filamentous feltwork. Zeiotic processes are marked *Z*. × 28,600.

FIGURE 6 *b* Free side of a HeLa cell in monolayer after 1 h of treatment with 0.25 Mg/ml of CD. A skein of microfilaments constitutes a thick (0.25–0.35 μm) subplasmalemmal pad in the cortex. A few parallel arrays of thin microfilaments arranged in small bundles (between brackets) remain intact within the wide microfilament mesh. The nucleus (*N*), a multivesicular body (*mvb*), and a vesicle (*v*) joined to plasma membrane are indicated. Sections of zeiotic protrusions are marked *Z*. × 45,500.

of the patterned binding of heavy meromyosin (HMM) to them. That the thin 5 to 7-nm filaments of the cell cortex can bind HMM in absence of ATP, and other evidence that they are actin, is now well-established for many cell types. The thin microfilaments of the ectoplasm of HeLa and Vero cells also bind HMM in repeating pattern; both their number and parallel ordering seem to be increased by the application of HMM, as noted also by Holtzer et al. (25). The presence of 0.25–0.5 μg/ml CD during glycerination of the model preparations and their exposure to HMM is without effect on the binding of HMM by cortical filaments or on their arrangement (Figs. 8, 9). Exposure of living HeLa cells to 0.25 μg/ml CD for 30 min before glycerination yields preparations in which the skeins, i.e. feltlike filament masses, are clearly recognizable in the cortex (Figs. 10, 12). Application of HMM to such models shows that most of the filament segments in those masses have the capacity to bind HMM (Figs. 11, 13, 14), although some, especially in the interiors, appear uncomplexed (Figs. 13, 14).

The Cortex after Prolonged Exposure to CD

The filament masses are remarkably durable in the continued presence of moderate concentrations of CD. In HeLa or HEp2 cells exposed to 0.25–0.5 μg/ml for up to 72 h, most of the compact feltlike material of the free side, is gathered under the apical 'bouquet' of zeiotic processes (Figs. 5, 7). However the subplasmalemmal cytoplasm of part of the free surface peripheral to the central convexity is almost devoid of filament meshes. This would appear to have resulted from a continued centripetal migration of filament skeins into the apex.

The central part of most of the cells remains more convex as though bunched or in a continuing state of contracture while in CD. But the peripheral cytoplasm of some of these cells appears to spread and flatten beginning approximately at 6–8 h, and continuing over the ensuing 18 h. These cells however do not regain their original planar area (Godman et al., manuscript in preparation). Such

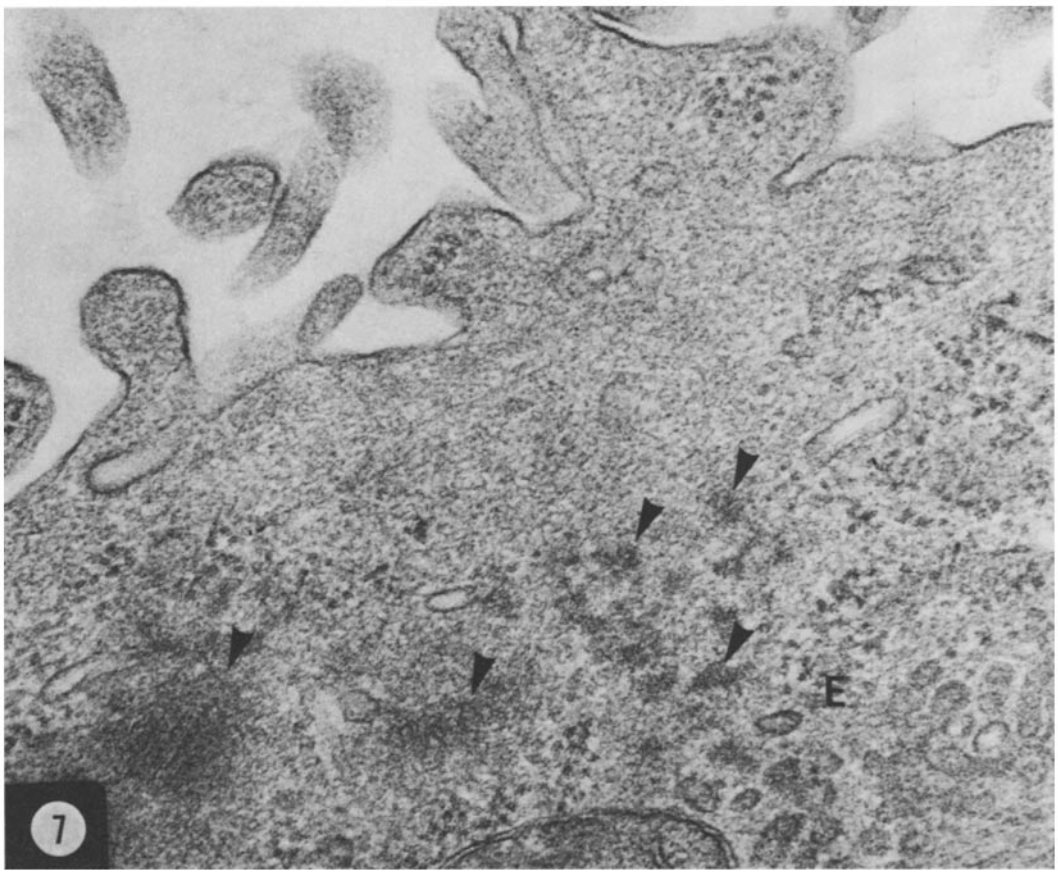


FIGURE 7 The free side of a HeLa cell exposed to 0.25 μg/ml CD for 6 h. A compact skein of kinked and ravelled thin microfilaments is accumulated in the cortex in a large (0.35 μm thick) mass from which endoplasmic contents (*E*) are generally excluded. In thin sections only short segments are visible. Within this filamentous mass, near its subcortical aspect, there are patchy densities (arrowhead). The larger, less compact densities consist of thick, rather straight filament segments embedded in a somewhat dense matrix; in the patch at lower left these filaments are stacked in parallel. The smaller denser masses at right show less internal detail; they have been interpreted as Z-like bodies. × 66,000.

cells, with few microextensions, have straight simplified outlines and polygonal or discoid shapes. Unlike the normally extenuated cell in monolayer, much of the peripheral cytoplasm at the edges of the cell reflattened while in CD does not have a normal ectoplasmic cortex: the web of thin microfilaments is either very thin or apparently absent. Here, as in the zeiotic protrusions, endoplasm may appear to abut directly on the inner face of the plasma membrane. Surface specializations, i.e. microvilli or smooth pinocytic vesicles, do not form in these areas, the surface of which remains smooth ("bald") while the cells are in CD. Even after prolonged treatment with CD, both the 10-nm tonofilaments of the endoplasm and the microtubules are numerous and well-preserved (Figs. 15, 18). Sparse arrays of thin microfilaments can still sometimes be discerned in microextensions and at the basal side (Fig. 17).

A proportion of the population that remains contracted after long periods in CD may develop mixed filament bundles or compact filament bundles of high density (Fig. 18). Small formations of this kind are evident in rare cells even within 2–4 h after exposure to moderate doses of CD (Fig. 6), but they do not become prominent until after 18 h, and then in only relatively few of the HeLa cells, but in many more MDBK cells. These bundles

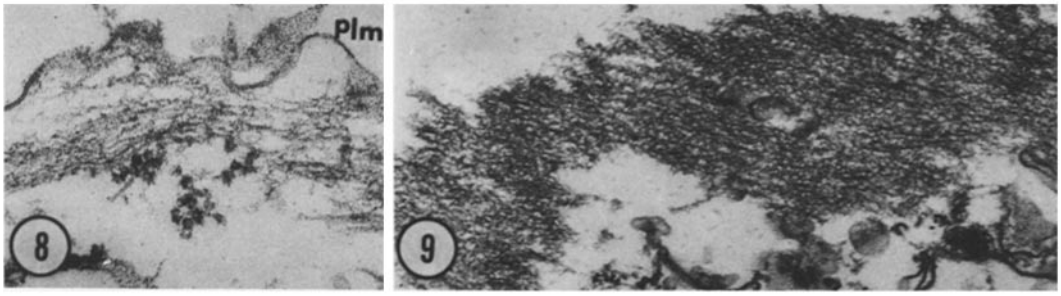


FIGURE 8 The cortex of a HeLa cell glycerinated as described in Materials and Methods. A bundle of arrayed thin microfilaments, as well as some filaments of the meshwork, are shown subjacent to the plasma membrane (*plm*). × 73,500.

FIGURE 9 Cortex of a glycerinated HeLa cell after exposure to heavy meromyosin (see Materials and Methods). The heavy meromyosin is bound to the thin microfilaments in a repeating spiky pattern, indicative of the formation of actin-myosin complexes. The resultant filament arrays are more closely packed. This picture is indistinguishable from those obtained using HMM preparations pretreated with CD (0.5 μg/ml for 30 min) or those in which HMM is applied to extracted cells in presence of CD (0.5 μg/ml). × 73,500.

consist of parallel arrays of the thin (about 5–7 nm) microfilaments, together with variable number of thicker (8–15 nm) very dense filaments (or filament segments). A regular stacking of filaments is not observed. In addition to these mixed bundles, some of the continuously treated cells accumulate very dense, compact, often tapered ribbonlike fibrils; these lie chiefly in the subcortex (Fig. 18). They measure from 0.4 to more than 1.0 μm in length and most are about 30–80 nm in greatest width. These bodies and those in Fig. 20 are similar in appearance to those hitherto described in other circumstances, as myosin aggregates or ribbons containing myosinoid filaments (q.v.). Such fibrils are more frequent in MDBK cells than in HeLa, and have not been observed in Vero.

Recovery

After treatment with moderate doses of CD, restitution of form and function begins soon after withdrawal of the agent and in most cells is essentially complete in 2–3 h, as judged in the phase microscope. Recovery is more protracted after long exposures or higher concentrations of CD. During the first 4 h after withdrawal of CD, some of these cells continue in a state of partial, albeit diminishing, contracture until completion of recovery. In such cells, large, well organized longitudinal bundles of thin microfilaments may occupy much of the cortex especially at the underside of the cell (Fig. 20). They are more extensive and better developed than the filament arrays of untreated cells. Most striking are the close-packed, obliquely oriented filament arrays often constituting a sheath in the basal cortex; they may attain a thickness and luxuriance not seen in control preparations (Fig. 21). Both the fascicular and sheathlike arrays may include patches of mixed thick (8–15 nm) and thin (5–7 nm) microfilaments (Figs. 20 and 21). Some of the former appear 'spiky' because of an apparently periodic accentuation of density, resembling somewhat the meromyosin-decorated elements of experimental preparations. In addition to these arrays in some of the recovering cells, clusters of the dense tactoidal or ribbonlike bodies tentatively designated 'myosinoid' aggregates lie in the endoplasm (Fig. 20).

At the top side of the cell, a continuous network of thin microfilaments underlying the plasma membrane is reconstituted soon after withdrawal of moderate doses of CD. But even after 2 h this mesh forms a layer in many places deeper or wider than normal (Fig. 22). Circumscribed, apparently spherical aggregates of compacted filamentous material are sometimes encountered in the subcortex and endoplasm of occasional partly recovered cells (Fig. 23). Most of the filaments composing these masses are thicker (10 or more nm) and denser and have irregularly lumpy profiles.

Cellular Response to High Dosage with CD

In the continued presence of rather high but subtoxic doses of CD (e.g. 0.5–1.0 μg/ml for

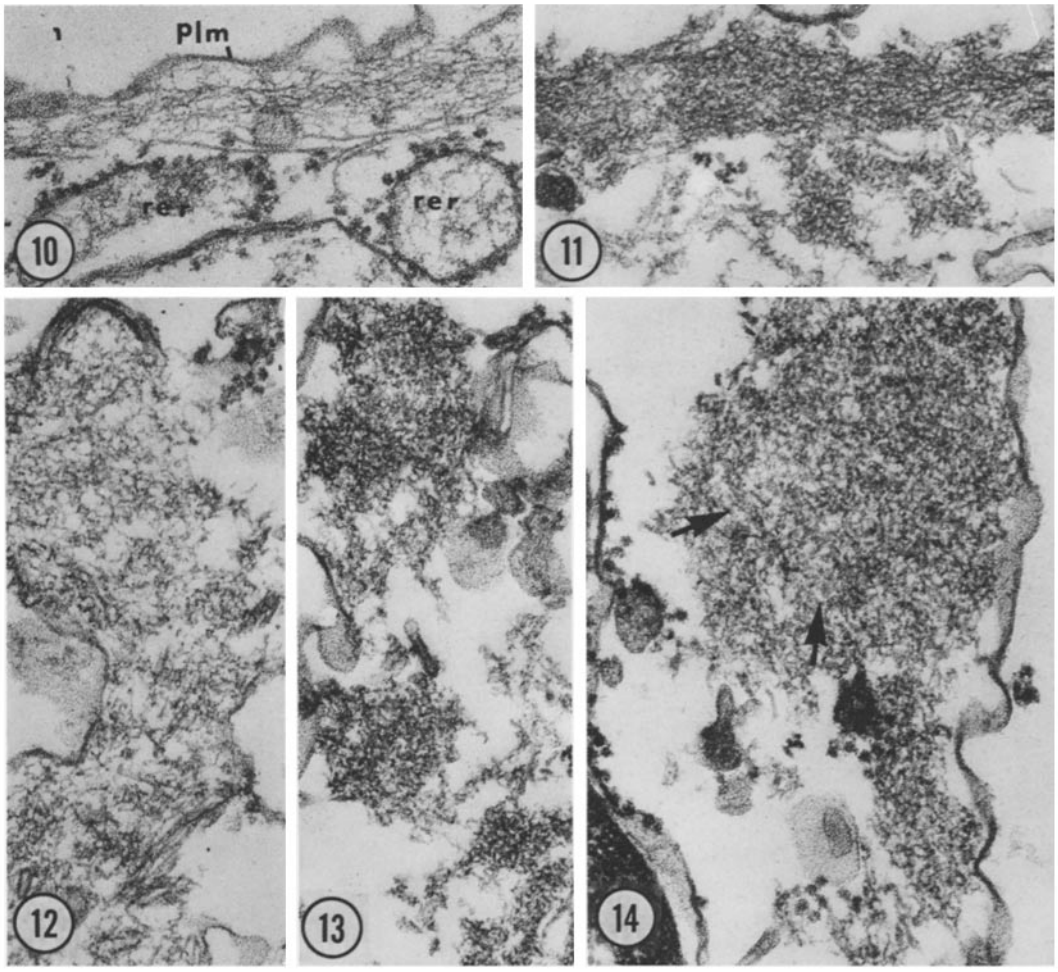


FIGURE 10 Cortex of a HeLa cell exposed to CD (0.5 μg/ml for 30 min) during life and then glycerinated. A bundle of thin microfilaments (and some elements of a filament meshwork) is evident in the cortex under the cell membrane (*plm*). Segments of rough endoplasmic reticulum (*rer*) are seen in the subjacent endoplasm. × 73,500.

FIGURE 11 Sector of a HeLa cell treated with CD (0.5 μg/ml for 30 min) during life, then glycerinated and subsequently exposed to heavy meromyosin (HMM). Patterned binding of HMM in packed filament arrays indicates unimpaired formation of actin-myosin complexes. × 73,500.

FIGURE 12 Sector of a HeLa cell prepared as in Fig. 10 in which unarrayed microfilament meshworks (feltlike masses) are gathered in the cortex. × 73,500.

FIGURE 13 Sector of a HeLa cell prepared as in Fig. 11 illustrating the binding of heavy meromyosin to the microfilaments of the skeins of fleece formed in the cortex under the influence of CD. × 73,500.

FIGURE 14 Cortex of a HeLa cell prepared as in Fig. 11. A large feltlike filament mass is gathered under the cell membrane. Heavy meromyosin appears to be bound to most microfilament segments of the skein, but in some areas (arrow), especially toward the endoplasmic side, filaments have remained uncomplexed. × 73,500.

HeLa; 2.0 μg/ml for MDBK cells) for 5–6 h, the total amount of microfilamentous fleece appears to be greater than that evident after exposure to lower doses; it may occupy areas extending inward as far as to the cell center. At about this time in the regions just under or between the filament masses, smooth membranous saccules and vesicles, evidently originating in endoplasmic reticulum, become adlineated and are sometimes seen apparently in course of fusing to form elongated saccules (Fig. 15) which can demarcate and in unusual instances ultimately separate the filament masses from the subjacent cytoplasm. With high doses (e.g. 2–5 μg/ml for HeLa), feltlike masses may be partly sequestered by these membranous elements and are sometimes sloughed at the surface. Accumulation of endomembranes about the CD-induced filament masses in a sequence akin to the early stages of autophagy suggests that persisting filamentous material of the compact masses may be recognized by the cell as 'foreign' elements.

DISCUSSION

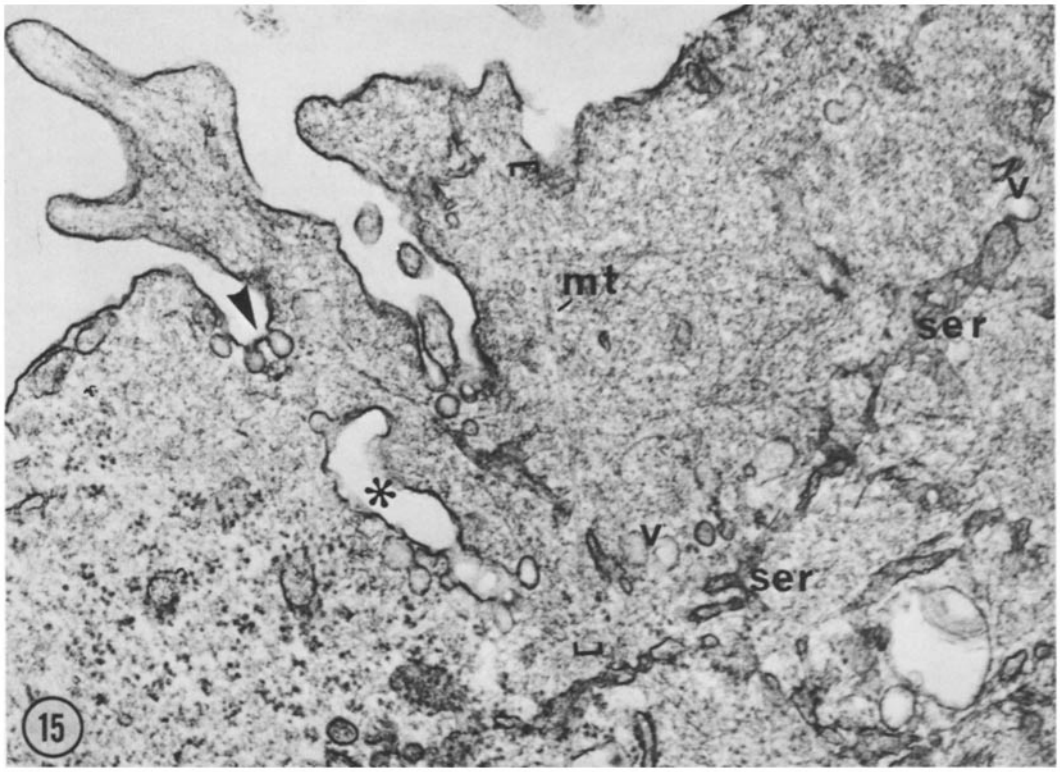
The Contractile Apparatus

It is generally agreed that the 5 to 7-nm microfilaments of the cell cortex [α-filaments (32)] whether arrayed or in nets are (or contain) actin (9, 11, 12, 17, 26, 33, 42, 49, inter alia.) The microfilaments presumably participate in the contractile function by interacting with myosin, [which, like actin, is probably of general distribution (for references see: 36, also 5, 7, 57)]. Although the degree of aggregation and the localization of the myosin in such nonmuscle cells is in doubt, and the nature of the regulatory proteins is uncertain, contraction is thought to depend upon the sliding of anchored actin filaments on myosin (30, 41). In nonmuscle cells myosin is mostly dispersed and may discharge its function by transitory aggregation into oligomers or multimers (30).

The traction of microfilaments of the cortical network, which are attached to the plasma membrane (10, 41) by some as yet unknown connection, probably effects membrane movements. The meshworks of actin microfilaments gives consistency to the cortical gel, and also brings about the local contractions of the ectoplasm that generate the motive forces for translatory movements (see 30). The arrays of thin filaments, like the filament nets, are extremely labile structures undergoing continual assembly, disassembly, and reorganization, even when protein synthesis is inhibited (15, 22). The evident alignment of the filament bundles in directions of streaming and of physical stress (19, 30, 40, 58), and in microprocesses (22, 60) the close spacing of filament arrays to form the sheath of the basal side of extended cells, their condensation at foci of adherence, and their stability during rapid movements, suggest that they are structural or skeletal elements. However such fibrils or filament arrays are also a feature in situations in which motive force is generated (see 30), suggesting that filament-arrays also have some function in contraction. The appearance of thin filament arrays, sometimes in association with thick (15–25 nm) myosinlike filaments during cytoplasmic contraction (13, 30, 40, 58, 62) and their shortening during the development of maximal tension (58) indicate that at least some such fibrils may be active in the generation of force. Although fibrils (i.e. organized filament arrays) may not be necessary for contraction, since this function is apparently capable of being performed by the filament reticulum, it has been suggested that they may serve to augment motive force when more powerful local movement is required (1, 30). Perhaps the arrays of thin filaments such as the 'myoid' fibrils depicted in cytochalasin B (CB)-treated baby hamster kidney (BHK) cells by Goldman (20) were functional as contractile elements, but that other bundles consisting apparently only of thin filaments might be actively contractile, seems less obvious.

An Interpretive Reconstruction of the Apparent Sequence of Events Occurring during Exposure to CD

Concomitant with the onset of CD-induced contraction (36) some of the thin microfilaments of the cortical mesh are displaced; they congregate in larger, more condensed packets (Fig. 4 *a*). This may entail detachment from their connections with the cell membrane. Sectors of the subplasmalemmal cortex may thus be largely denuded of a microfilament web permitting the incursion of endoplasm to the undefended inner face of cell membrane. Intracellular tension which accompanies contraction of the cortical gel, transmitted centripetally, expresses these endoplasmic plugs outward, causing them to protrude (zeiosis). Compaction of the skeins of filaments and their coalescence gives rise to the big feltlike pads and masses.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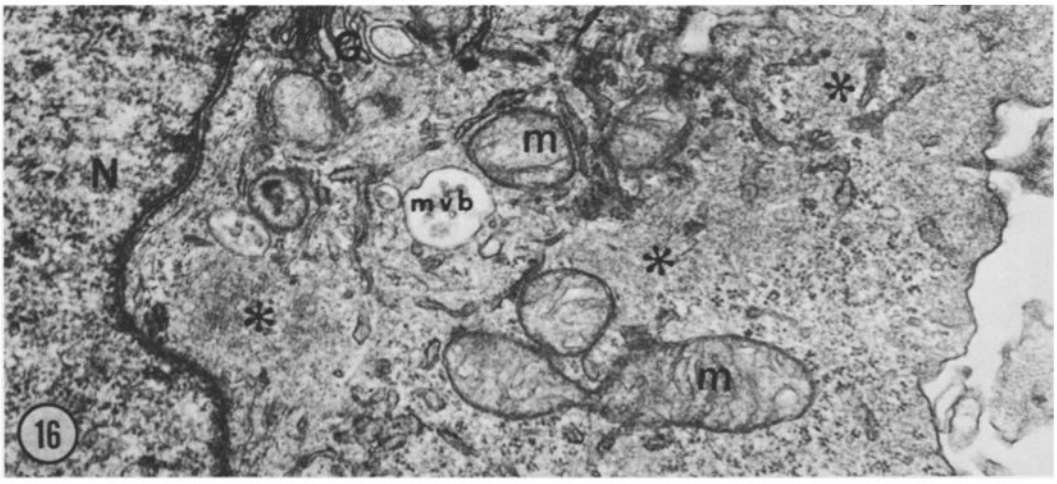
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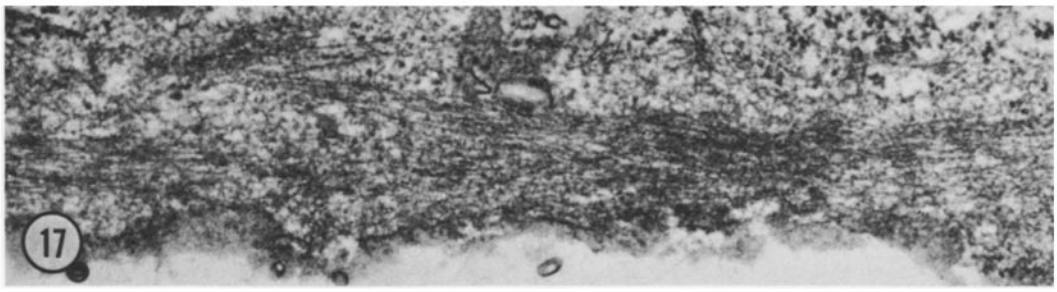

17

The pressures promoting zeiotic extrusion may also draw some filament-fleece into the peduncles of the knobs and the underlying cytoplasm (Figs. 5, 7). The migration of zeiotic knobs and their congregation at the apex (34) is accompanied by a similar movement and aggregation of their subjacent filament skeins which eventually gather in masses under the apical zeiotic 'bouquet.'

Most filament arrays are disassembled. Some arrays, however, either persist or are reorganized during 5–6 h of exposure to effective doses of CD (Figs. 6 *a–b*, 17).

The appearance of the cortex after some 8–12 h in CD, when some apparent spreading out of most hitherto retracted cells begins, is not like that of normal cells in process of settling and spreading (Miranda et al., manuscript in preparation). The small amount of apparent respreading in presence of CD evidently results from centrifugal flow of endoplasm which is no longer contained at the periphery by a competent ectoplasmic gel. This isodiametric flux of endoplasm resembles an early phase in the extension of pseudopodia (33).

Within an hour after withdrawal of CD, a continuous microfilament web is reconstituted in the cortex, and impressive parallel arrays of thin filaments with many dense zones make their appearance. These formations would appear to be important for normal stretching out and regaining normal shape and motor function.

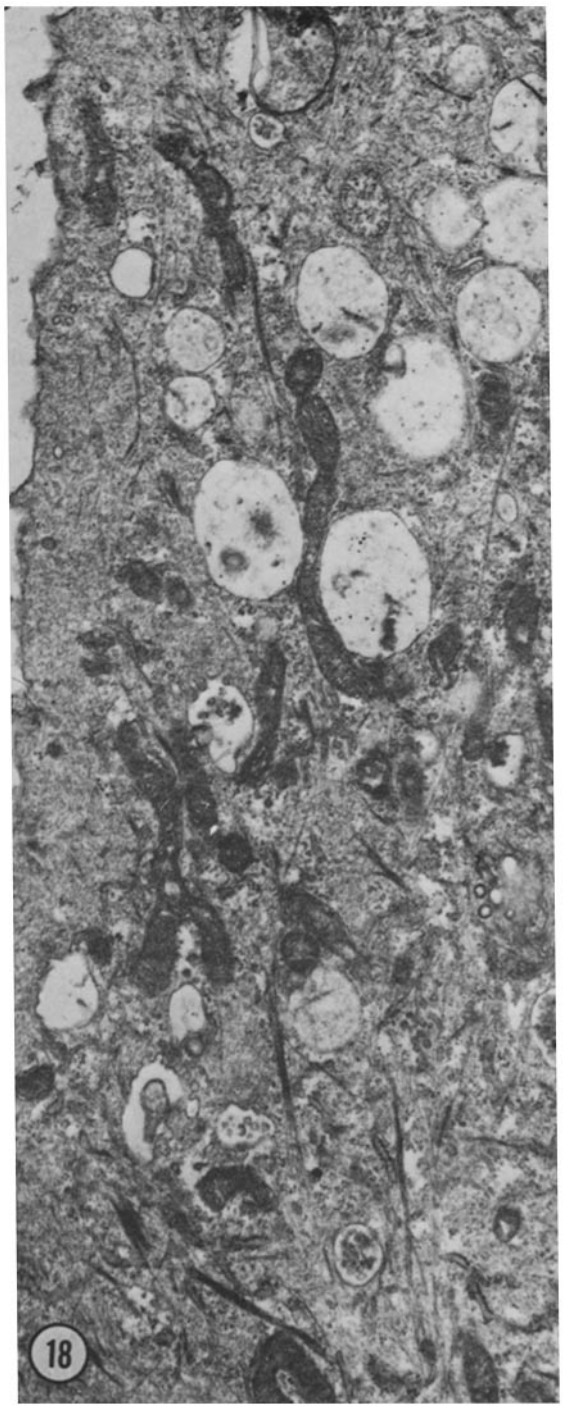
Microfilament Skeins

Accumulation and condensation of thin microfilaments in feltlike skeins is progressive with continued exposure to CD. Their subplasmalemmal location and physical continuity with still unaltered cortical microfilaments early after application of CD, and their growth at the apparent expense of the normal filament meshes, leave little doubt that they form, at least initially, by the aggregation and packing of filaments of cortical networks. In thin section, the feltlike masses are mostly resolvable into short profiles 5–8 nm thick which can be interpreted as filament segments of a compacted, highly ravelled skein cut in random planes. Masses of this kind, formed under the influence of CB (8, 14, 31, 52, 53, 61, 62, 63, inter alia), have been regarded as accumulations of 'disaggregated' filament materials, a view consonant with the widely held hypothesis that CD may 'disrupt' certain classes of thin filaments (3, 53, 54, 63). However, the difficulties of resolving the feltlike material have led some of the same investigators to suggest that, alternatively, the masses might represent a 'contracted' form of the filament network (53). That the feltlike masses gathered under the influence of CD are, indeed, ravelled, compacted, and displaced but physicochemically intact thin filaments of the cortical reticulum, is suggested by the morphological sequences and

FIGURE 15 Sector of free side of an MDBK cell after 5.5 h of exposure to 2.0 μg/ml CD. A skein of microfilaments is gathered in a feltlike mass that occupies the cortex. Some long relatively straight microfilaments of about 6.5 nm course intact through the filament meshwork. A long microtubule (*mt*) is indicated between brackets. At the extreme left, where the skein appears to be interrupted, endoplasmic contents approach the plasma membrane. Numerous vesicles (*v*) and cisternal segments of smooth endoplasmic reticulum (*ser*) lie chiefly at the borders of the filament mass. The vesicles and segments of *ser* appear to be fusing to form elongate cisternae such as that marked. The cluster of smooth vesicles is joined to plasma membrane at the bottom of the cul-de-sac shown by the arrowhead. × 45,000.

FIGURE 16 Sector of the abbasal side of an MDBK cell after exposure to 2.0 μg/ml of CD for 5.5 h. Large connected masses of microfilamentous felt (asterisks) occupy much of the cortex underlying the cell membrane (plm) and extend centrally, deep into the perinuclear endoplasmic area close to the nuclear envelope. Endoplasmic contents (at lower right) approach close to the plasma membrane but are still separated from it by a narrow microfilament mesh continuous with the main mass at upper right. The nucleus (*N*), mitochondria (*m*), endoplasmic reticulum (*er*), golgi lamellae (*G*), and multivesicular bodies (*mvb*) are marked as indicated. × 27,500.

FIGURE 17 Basal aspect of an MDBK cell after exposure to 2.0 μg/ml of CD for 5.5 h, sectioned somewhat tangentially. Arrays of thin microfilaments in bundles, such as this which lies parallel to the flat plane of the surface, are frequent in CD-treated MDBK. Patchy densities or dense segments are evident along the course of the bundles. × 40,000.



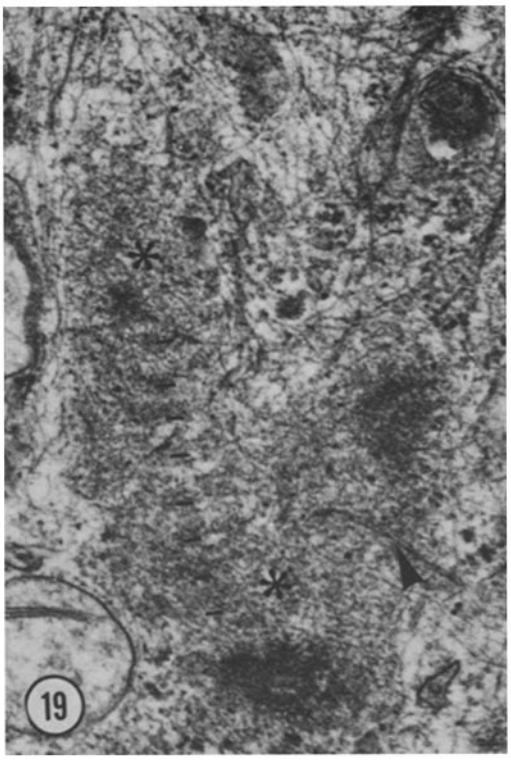
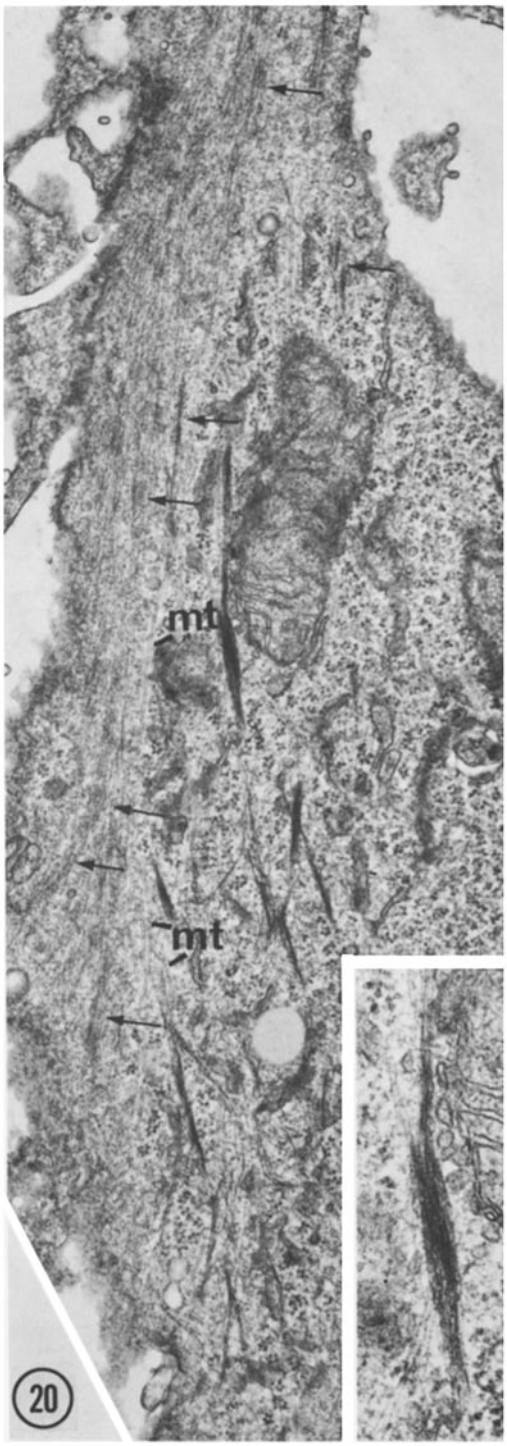


FIGURE 18 Segment of an MDBK cell exposed to CD (2 μg/ml) for 24 h. The cortex and subcortex are occupied by microfilamentous felt, masses of which extend deep into the endoplasm (asterisk). This area contains dense ribbonlike fibrils or fibers consisting of tight microfilament bundles. The numerous dilated vacuoles, multivesicular bodies (*mvb*) and other lysosomes which beset the area, and the small foci of cytoplasmolysis (arrowheads) are signs of some toxicity. × 18,000.

FIGURE 19 Cytoplasm of an MDBK cell exposed to 2 μg/ml CD for 24 h. Filamentous material is accumulated in bulky masses (asterisk) within which large dense patches (and a slight suggestion of periodicity) have developed; these are probably Z-like bodies. × 50,400.

their capacity to bind HMM. Their appearance in thin section might be compared with that of the coiled and highly ravelled chromatin in the interphase chromosome. They are remarkably similar to the condensed "granulo-fibrillar" filament masses observable in the cytoplasm of amoebae and slime molds after treatments producing marked local contraction of cytoplasm (30), and

especially to those seen in glycerinated models of such varied objects as actomyosin threads, amoeba, slime-mold protoplasm, or fibrocytes and leiomyocytes induced to hypercontraction by ATP (4, 30, 48). Somewhat similar filament skeins are sometimes seen in pathologically contracted cells. It is noteworthy that under these conditions of extreme contraction thick myosinoid filaments or aggregates may also be induced in models (4, 30,



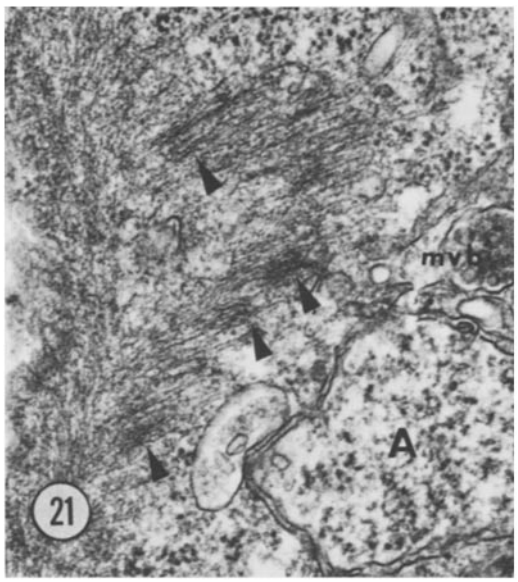


FIGURE 21 Sector of the base of a HeLa cell after 1 h in fresh medium after exposure to 2 μg/ml of CD for 3 h. The cortex in this oblique section is occupied by an unusually thick sheath of microfilament arrays in which there are "dense" patches containing thick filament segments (arrows). A large autophagic body (*A*) and a multivesicular body (*mvb*) are at upper left. × 45,000.

FIGURE 20 Tangential section of a HeLa cell treated with a high dose of CD (2 μg/ml for 3 h) and allowed to recover in drug-free medium for 2 h. Longitudinally oriented bundles of thin (5–7 nm) microfilaments in parallel array course along most of the length of the cell. At their termini the filaments are apparently connected with plasma membrane. At irregular intervals the fascicles exhibit small denser patches (arrows) within which there are also filaments of greater thickness and density. Numerous long microtubules (*mt*) are oriented in parallel with the filament bundles and in close spatial relation to them. In the subcortex and deeper endoplasm there are many dense ribbonlike or tapered fibrils (up to 1 μm long; 30–80 nm wide); these are tentatively considered to be myosinoid aggregates. × 25,000. One of these dense fibrils is shown at higher magnification in the inset. × 55,000.

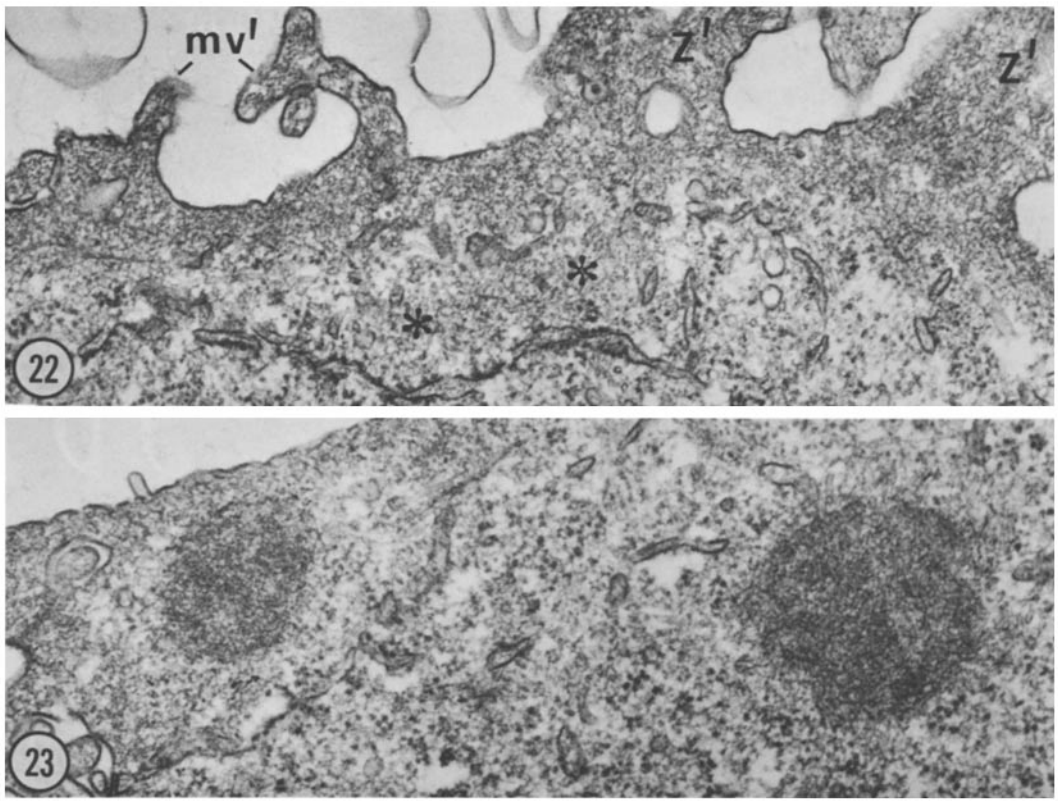


FIGURE 22 Top side of a partly recovered HeLa cell after 2 h in fresh medium after exposure to 0.5 μg/ml of CD for 2 h. A continuity of the cortical microfilament meshwork has been restored; it forms a thicker than usual (0.16–0.4 μm) layer under the cell membrane with extensions into the subcortex (asterisk). Microvillous processes, abnormally blunt and thick, are indicated by *mv;* the large wide extensions filled with microfilament mesh represent the rests of zeiotic processes (*Z*). × 37,750.

FIGURE 23 Subcortex of another region of the same cell as that represented in Fig. 22 containing spherical masses composed chiefly of skeins of thick (> 10 nm) dense microfilaments. They may be residua of myosinoid aggregates. × 37,750.

48, 58). Ravelling and condensation of the thin microfilament reticulum would thus appear to be characteristic of the hypercontracted state. Their compaction in feltlike skeins need not, indeed probably does not, entail a basic change in the conformation of the actin filaments akin to disruption or disaggregation. The rapidity with which the cortical reticulum of thin filaments is reconstituted after withdrawal of CD, at the apparent expense of the feltlike masses even during the inhibition of protein synthesis by cycloheximide (Godman et al., manuscript in preparation), further suggests that the filaments, although redistributed, had not suffered structural injury.

The inhibition of sugar transport chiefly by CB across the plasma membrane of some cell types, which has sometimes been adduced as an indication that cytochalasin may act primarily at the cell surface, is unrelated either to cell contraction (36), changes in shape (29), or to the formation of filament masses (59, 64). In any case CD does not significantly curtail hexose transport in cells of most of these lines and the 45% reduction of hexose uptake by CD in HeLa appears to be without important functional consequence (36). Whether CD exerts its effects directly on the contractile proteins or on their regulatory apparatus, or indirectly via a membrane system, the resulting structural change affecting the thin filaments can be regarded as a rearrangement that occurs pari

passu with the hypercontracted state.

Filament Arrays

Although the arrays of thin microfilaments that constitute the bundles and the cortical sheath of the base (and sides) of the cell are relatively less sensitive to the effects of CD than are the filament nets, with longer exposure or high concentrations most of them disappear, apparently to be replaced by the filament feltwork. Some of the dense patches of the arrays which include thicker filament segments suggest a more organized association of contractile proteins: in any case such dense segments are somewhat more durable in presence of CD.

Filaments of purified actin, reportedly subject to deformation by high concentrations of CB, are protected after complexing with troponin-tropomyosin (54). The inhibition of ATPase activity of myosin by CD does not occur in actomyosin complexes (43). Orderly arrays of actin and myosin within myofibrils are unaffected by CD (35). These examples may illustrate the greater resistance of interacting complexes at various levels of organization to alteration by CD. The rate of decay of filament bundles in presence of CD is roughly dose dependent. The reported intactness of the filament sheath (53) and even of the cortical nets (22) after doses of CB that stop membrane ruffling and cell locomotion might reflect the lesser potency of CB as compared with CD. The filament bundles of cell types like MDBK, endowed with more highly organized arrays are also relatively more resistant to the effects of CD.

The remarkable mixed "musclelike" bundles described by Goldman (20) in some BHK cells after 24 h of CB were organized as regularly alternating thick and thin filaments apparently joined by cross-links like those of myofibrils; also depicted were rare leptomeric bodies such as are induced in abundance by CD in myocytes (35). These musclelike bundles of BHK cells (20) and the mixed bundles and myosinoid aggregates depicted in Figs. 18 and 20 occur only in contracted cells. It may be significant for understanding the genesis of these myoid bodies in nonmuscle cells during treatment with CD, that fibrils composed of associated thick (myosin) and thin (actin) filaments develop regularly in the cytoplasm of amoeba or of slime mold where local or general cytoplasmic contraction has been induced, whether in the intact organism, in preparations of cytoplasm stimulated by ATP or Ca^{++} (19, 40, 58, inter alia), or in glycerinated preparations contracted by ATP, and even more remarkably, in ATP-contracted actomyosin gels (see 30). More random but similar heaps of thin and thick filaments occur in glycerinated fibrocytes and smooth muscle induced to contract ATP (4, 48).

Although it is now generally held that thick filaments of organized myosin are a stable component of leiomyocytes (18, 24, 44, 50), their occurrence in physiological conditions was once called into question because of their apparent lability and their apparent absence, especially in such states as relaxation or after fixation procedures (28, 38, 39, 47). Nevertheless, more numerous thick filaments and longer ribbonlike aggregates of myosin are elicited in smooth muscle in various circumstances that promote contraction (24, 47, 51) or mechanical tension (18, 51) and after slower procedures of fixation, that may tend to cause contraction (30, 51). Fibres of fusiform shape, called myosinlike, have also been produced in smooth muscle by trypsinization (45). In platelets, which also have a contractile function and are rich in myosin (2, inter alia), thick filaments and typical large tactoids, presumably of myosin, form readily when platelets are osmotically shocked, or glycerinated and incubated with Mg-ATP (6, 65) or trypsinized (46). In every case some manipulation which tends to bring about supravital or agonal contraction without subsequent relaxation (i.e. contracture) would seem to favor the aggregation of myosin or a myosin component not only into thick filaments but also into larger dense bodies of characteristic appearance.

In some cells in which hypercontraction is long sustained by the continued presence of cytochalasin, thick filaments or myoid bodies like those described above also appear in the living cytoplasm [see also (20)], and in view of the circumstances of their elicitation in the foregoing models it can be inferred that marked or prolonged contraction may somehow be causally related to the aggregation of myosin which may normally be present only in oligomeric or multimeric form.

Thin filaments and dense aggregates may persist into the recovery period until true physiological respreading has been completed; during this time elaborate thin filament bundles and sheaths occur (Fig. 20). The extraordinary development of these thin filament arrays, permitted by withdrawal of CD, appears to be related to respreading and the resumption of cell movement and their attendant

deformations of shape.

Hypotheses

Neither the locus nor the mechanism by which CD effects the contractile apparatus of cells is known. Current hypotheses, more or less at variance, favor: (*a*) a site of action at the plasma membrane based mostly on the inhibition of hexose transport by CB in some cell types (see 36 for review). To this must be added the observation that tritiated CD is bound chiefly to plasma membrane (56), and the hypothesis that CD may act on the contractile proteins by affecting primarily the function of a membrane system must therefore be entertained. (*b*) A direct effect on actin based chiefly on an interpretation of fine structure of treated cells (8, 53, inter alia) and on the deformation of pure actin filaments in vitro by CB (54). (*c*) An effect on actomyosin, manifested by diminution of its Mg-ATPase activity in presence of CB (37, 55). (*d*) An effect on myosin, as indicated by the CD-induced inhibition of myosin ATPase activity (but not that of actomyosin), and by the reported binding of radioactive CD to myosin (43). If some myosin were membrane-associated (7) this action of cytochalasin could be reconciled with the first hypothesis.

The components of the contractile apparatus are in a dynamic state, continually assembled and disassembled and associated and dissociated during the contraction-relaxation cycle. If it is assumed that actomyosin complexes existing at the time of application of CD are induced to shorten, and in the continued presence of CD prevented from dissociating, then as new contractile complexes of actin and myosin are recruited from the pool of unassociated elements a sustained and even increasing state of contraction (contracture) would ensue. As indicated in descriptions of the various experimental models (actomyosin gels, isolated cytoplasm, glycerinated preparations of living cells), the condition of extreme contraction without subsequent relaxation is accompanied by compaction and displacement of thin filaments in masses, the appearance of thick myosin-like filaments, and in some instances, larger bodies. These are, in effect, the changes that we have described in the contractile apparatus of living cells exposed to CD. The compression and disorientation of the filaments that accompany their displacement and compaction under the influence of CD might perhaps be compared to the state of the myofilaments during the supercontraction of muscle. The diminishing rate of contraction in the latter part of the period of active retraction induced by CD (36) may be due to "locking in" (i.e. nondisjunction) of increasing numbers of functional elements. This postulated action of CD on interacting contractile proteins could also be invoked to explain such phenomena as its reported effects on exocytosis and secretion (for citations see 36) if the membranes effecting these processes were linked with myosin in the manner proposed by Berl et al. (7).

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Note added in proof: In the preceding paper of this series (Miranda et al. 1974. *J. Cell Biol.* **61**:481.), a reference to the effect of CB on hexose transport in HeLa cells was erroneous. In fact, transport of glucose or 2-deoxyglucose into HeLa is completely inhibited by CB at about 3 μg/ml (Mizel, S., and L. Wilson. 1972. *J. Biol. Chem.* **247**:4102); however, CD inhibited uptake by only about 50%.

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