ACTION OF CYTOCHALASIN D ON CELLS OF ESTABLISHED LINES

I. Early Events

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ABSTRACT

HeLa, Vero, L, HEp2, and MDBK cells respond immediately to 0.2–0.5 μg/ml cytochalasin D (CD) with sustained contraction (contracture), loss of microvilli, expression of endoplasmic contents (zeiosis), nuclear protrusion, and extension of cytoplasmic processes. The development of these changes is depicted, and the dose-response patterns in these cell lines are described. MDBK is generally most resistant and HeLa most sensitive to these effects of CD. Cells in G_1 are most sensitive to CD; responsiveness decreases progressively during early S and is least in mid S through G_2. CD inhibits transport of [^{14}C]deoxyglucose in HeLa by about 45% but has no significant effect on hexose uptake in Vero and MDBK; sugar transport is thus apparently unrelated to any morphologic effect of CD. Although spreading and attachment are impeded, CD does not decrease and may even enhance the adhesiveness of established monolayers. Contraction appears to be a primary early effect of CD, upon which other visible changes follow. It is prevented by some inhibitors of energy metabolism (deoxyglucose and dinitrophenol) and does not occur in glycerinated models without ATP. The possible bases of the contractile response to CD are discussed. Although direct or indirect action of CD on some microfilaments may occur, a generalized structural disruption of contractile filaments by CD is considered unlikely.

INTRODUCTION

Agents that have more or less specific cytopharmacologic effects are powerful tools for studying the functions of cellular organelles. The cytochalasins were thought to be among those drugs that have defined effects on specific organelles. Since most of the consequences of exposure to cytochalasin could be referred to an interference with contractile movements of cells, presumed to be mediated by microfilaments, and because alterations in the arrangement of microfilaments themselves were depicted, it was postulated that cytochalasin acted by "disrupting," "disorganizing," or otherwise incapacitating a class of thin microfilaments in the cell cortex (18, 103). These effects of cytochalasin included, inter alia, flattening and "relaxation" of cells, (whence the name cytochalasin), paralysis of locomotion and of membrane movements (17, 35, 86, 103), expulsion

of the cell nucleus (17, 74), prevention of cytokinesis (17, 35, 81), suppression of pinocytosis (6, 95) and of phagocytosis (6, 26, 57, 114), interference with platelet function and clot retraction (83), prevention of morphogenetic movements in epithelia (8, 56, 85, 110), of axonal growth (111), cell sorting (89), suppression of cytoplasmic streaming (11, 106), inhibition of secretion (14, 63, 80, 105), blocking of the interaction of myosin and actin in vitro (88), the assembly of myofibrils, and contraction of cardiac muscle cells (58).

However, further study has revealed significant exceptions: cleavage furrowing, although not completed, is not prevented by cytochalasin (9, 31, 38, 53); contractility of myotubes is not diminished (61, 79), indeed, spontaneous pulsation is increased (61); organization of myofibrils proceeds in the presence of cytochalasin (61, 79); secretion is not necessarily blocked (65) and exocytosis may even be enhanced (27, 39, 41, 116); some forms of endocytosis are unaffected (49, 96, 108); and efficiency of infection with some viruses is augmented (28). Although the direct effect of cytochalasin B (CB) on actin fibrils in vitro is in some doubt (33, 87, 88), long treatment of cells with cytochalasin does not simply derange the cortical microfilaments (36). Indeed, many such microfilaments can become organized into impressive arrays[1] (36).

Moreover, the observation that CB can suppress the permeability of cells for hexoses (20, 23, 32, 51, 62, 115) and nucleosides (68), usually at concentrations lower than those required to induce other effects, could not readily be explained by a specific primary effect of this agent on microfilaments. Neither is it immediately apparent how most of the effects of this drug, which would seem to result from a disturbance in cellular contractility, can be reconciled with this primary action at the cell membrane. Similarly, the ability of CB to prevent the hormonal induction of tyrosine aminotransferase (15) may not be explicable in terms of a direct effect on contractile filaments without making additional assumptions that exceed exact information. In the present state of our knowledge then, there is no satisfactory or unitary explanation of the way in which cytochalasin works in cells, or of the primary site of its action. Such a rationale would be most desirable, and would certainly go far to elucidate the physiology of cellular movements, a subject of obvious importance in biology and pathology.

Almost all of the foregoing observations have been made with CB, the best known member of this group of six congeners. The other cytochalasins differ from B in potency, and may produce different effects in cells. Cytochalasin D (CD), the compound chiefly employed in the present studies like cytochalasin A (CA), differs from CB in its relatively limited capacity to interfere with hexose uptake in most cell lines,[2] but mole for mole it is more powerful than CB in provoking visible changes. Among the most arresting of the early effects brought about by CD at low concentration are contractile phenomena. Detailed attention has not hitherto been focused on these in descriptions of the effects of other cytochalasins. Closer study of the earliest manifestations of the action of cytochalasins can contribute toward an understanding of their mechanisms of action, as well as of the cell functions influenced by these agents. In this communication, the first events observable after application of CD are described. These include changes at the cell surface, and contraction of the cytoplasm and its consequences.

MATERIALS AND METHODS

Culture Methods

Monolayer cultures of HeLa (CCL2, American Type Culture Collection), HEp2, KB, L (NCTC clone 929), Vero, and MDBK were maintained in bicarbonate-buffered Eagle's minimal essential medium (MEM) supplemented with 10% (vol/vol) newborn or fetal calf serum, 60 μg/ml of streptomycin, and 60 U/ml of penicillin (Grand Island Biological Co., Grand Island, N. Y.). The cells were kept in exponential growth in an atmosphere of 5% CO_2 in air. Suspension cultures of HeLa S3 and L cells were maintained in Spinner MEM, supplemented as described above.

Synchrony

Synchronous cultures of HeLa were prepared by selective detachment of mitotic cells which were then allowed to proceed into G_1; in early G_1, when these cells had firmly settled, a 7.5-mM thymidine block was applied for 14 h, upon relief of which cells proceeded synchronously into S and subsequently parasynchronously to G_2. This procedure is a modification of the method described by Pederson and Robbins (67). The cells, suspended in medium conditioned by

[1] Miranda, A., G. Godman, and S. Tanenbaum. 1974. Submitted for publication. *J. Cell Biol.*

[2] Tannenbaum, J., A. Miranda, S. Sawicki, and S. Tanenbaum. In preparation.

exposure to HeLa monolayers for 24 h, were planted on 15-mm round cover slips in 24-well Linbro trays (0.8–1.2 × 10^5 cells/well) (Bellco Glass, Inc., Vineland, N. J.). The degree of synchrony and the phase in the cell cycle was checked by exposing cells released from blockade to 10 μCi/ml of [^{3}H]thymidine ([^{3}H]-TdR) for 10 min and counting the proportion of labeled cells in autoradiograms at the intervals indicated (Fig. 6).

Cytochalasin

CD, a metabolite of *Zygosporium masonii* (59), was purified (92) and dissolved at 1 mg/ml in dimethyl sulfoxide (DMSO). This was diluted with distilled water to make a stock of 100 μg/ml, which could be stored at −20°C. Final concentrations of from 0.01 to 8.0 μg/ml in culture medium were applied to cultures. Stock and working solutions of CB, generously supplied by Dr. S. Carter, were prepared in the same manner.

Inhibitors

Inhibitors of energy metabolism: 2-deoxyglucose (DOG), 2,4-dinitrophenol (DNP), sodium azide (NaN_3), and iodoacetate (IAA) (Sigma Chemical Co., St. Louis, Mo.), were dissolved in glucose-free MEM supplemented with 10% dialyzed newborn calf serum. Subconfluent monolayers on 22-mm^2 cover slips were rinsed with glucose-free medium and treated as described in Table I.

Models

Monolayers of HeLa and Vero cells were extracted with 50% glycerine at 0°C for 5–6 wk, as prescribed by Hoffman-Berling (43). Their contractile response to ATP was visualized in the phase microscope, and assessed quantitatively in Formalin-fixed, stained preparations by areal measurement (q.v.), the reduction of planar surface occupied being taken as a measure of the degree of contraction.

Microscopy

For examination of living cultures, cells were planted in modified Cooper dishes (29). Phase microscopy was performed with a Wild M-40 inverted microscope, equipped with an air curtain device (Sage Instruments Div., Orion Research, Inc., Cambridge, Mass.) for maintaining the temperature at 37°C. For counting the number of cells exhibiting expression of endoplasmic contents (zeiosis) and/or nuclear protrusion, subconfluent monolayers of cells (3 × 10^5 cells) grown for 24 h on 22-mm^2 glass cover slips in 35-mm plastic Linbro dishes (Bellco Glass, Inc.) were treated with CD or DMSO, fixed in 2.5% phosphate-buffered glutaraldehyde (pH 7.2), mounted in buffer, sealed, and examined with phase optics (E. Leitz, Inc., Rockleigh, N. J.). Preparation of cells for autoradiography and morphometry are described below.

TABLE I

Effect of Inhibitors of Energy Metabolism on Contraction Induced by CD in HeLa Cells

Inhibitor	Concentration	Percent inhibition of contraction
	M	
DOG	1×10^{-2}	100
DNP	5×10^{-3}	92
IAA	1×10^{-3}	<5
NaN_3	5×10^{-3}	<5

Monolayers of HeLa cells, preincubated with inhibitor for 6 min, were exposed to 0.2 μg/ml of CD (or 0.02% DMSO) in the continued presence of the inhibitor for 15 min. For each preparation the average area of 3,000–5,000 cells was determined by computer analysis (as described in Materials and Methods). Controls were treated with inhibitor and DMSO. The mean decrease in area occupied by the cells was compared with the areal change in cells treated with DMSO or CD alone, the decrease in area occupied by cells being a measure of their contraction. Results are expressed as percent inhibition of contraction.

For scanning electron microscopy, cell monolayers were fixed according to the method described by Porter et al. (72), dried by the critical-point method in a Denton DCP-1 apparatus, covered with gold-palladium in a Denton DV-502 evaporator (Denton Vacuum Inc., Cherry Hill, N. J.), and examined with a JSM-U3 scanning electron microscope, operated at 15 kV.

For transmission electron microscopy the cultures, grown in 30-ml flasks (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.), were fixed in 2% phosphate-buffered glutaraldehyde (pH 7.2), scraped off the surface and rinsed, osmicated, and stained in bulk with 0.25% aqueous uranyl acetate. The preparations were pelleted in agar (42) and dehydrated, embedded, and stained according to standard procedure (40). Sections cut with a Porter and Blum MT2 ultramicrotome (Ivan Sorvall, Newtown, Conn.), were examined with a Siemens Elmiskop I electron microscope.

Autoradiography

Subconfluent cultures (2 × 10^5 cells) on 22-mm^2 cover slips or synchronized preparations were exposed simultaneously to 0.2 μg/ml CD and 10 μCi/ml of

TABLE II

Effect of CD on Settling and Attachment

Medium	HEp2		HeLa		L		MDBK	
	Percent attached	Percent of control	Percent attached	Percent of control	Percent attached	Percent of control	Percent attached	Percent of control
DMSO 0.02%	—	—	61 ± 5	—	—	—	75 ± 5	—
DMSO 0.1%	42 ± 5	100	64 ± 4	100	37 ± 3	100	70 ± 4	100
CD 0.25 μg/ml	25 ± 4	59	39 ± 6	60	39 ± 6	105	79 ± 3	113
CD 1.0 μg/ml	17 ± 2	40	26 ± 3	41	32 ± 2	86	35 ± 5	50

Monolayers were prelabeled for 24 h with 1 or 2 μCi/ml [^{3}H]TdR (specific activity 29 Ci/mmol), washed, and incubated for 3 h in unlabeled medium. After trypsinization, cells were suspended in 2 ml of medium with either DMSO or CD and transferred to prepared glass scintillation vials. 2×10^5 cells were allowed to settle, and attach for 1 h at 37°C in a CO_2 incubator; the vials were then gently rinsed twice with EBSS and the residual radioactivity (representing attached cells) was determined as given in Materials and Methods. Results are expressed as the percentage of counts in the original inoculum: 2×10^5 cells (percent attached), and as the percentage of counts in attached DMSO-treated cells (percent of control).

[^{3}H]TdR (specific activity 29 Ci/mmol, Amersham/Searle Corp., Arlington Heights, Ill.), for 10 min. They were rinsed with Earle's balanced salt solution (EBSS), fixed, and prepared for autoradiography by dipping in a K-5 emulsion (Ilford Ltd., Ilford, Essex, England).

Area and Volume Measurements

24 hr after transfer, cell monolayers (3×10^5 cells per 35-mm dish) on 22-mm^2 cover slips, were treated with cytochalasin, DMSO, and/or inhibitors as indicated in figures. They were fixed in 10% neutral Formalin and stained with haematoxylin and crystal violet. Cell area measurements were carried out by systematic point counting (30), using a quadratic grid of lines intersecting at 1 cm on photomicrographs magnified to × 700. In some instances (Fig. 1 and Table I), the mean area occupied per cell was determined by computer analysis, using a Classimat image analyzer[3] (E. Leitz, Inc.), operated at × 300 on the television screen. The computer was programed to score the number of hits per cell (image points) and the number of nuclei per field.

For cell volume determinations, cells grown in suspension and trypsinized monolayers of CD- and DMSO-treated cells were counted with a model B Coulter counter (Coulter Electronics Inc., Fine Particle Group, Hialeah, Fla.). Thresholds were set to count the number of cells in 250 μm^3 class increments.

Adhesivity and Attachment

Adhesivity of CD- and DMSO-treated cells was determined according to the method outlined in the legend of Table III. The procedure for measuring cell settling and attachment, using [^{3}H]TdR is presented in Table II. The samples were solubilized with Soluene (Packard Instrument Co., Inc., Downers Grove, Ill.), and counted in a Packard Tricarb liquid scintillation spectrometer. The data were corrected for background. Controls showed that no loss of label ensued as a result of enucleation by CD under the conditions of these experiments.

Uptake of 2-[^{14}C]Deoxyglucose

24-h old cultures grown in 24-well Linbro trays (1×10^5 cells/well) were rinsed with glucose-free growth medium at 37°C. They were treated for 15 or 30 min at 37°C with medium (glucose free) containing CD and 2.5 μCi/ml 2-deoxy-D-[1-^{14}C]glucose (specific activity 54.4 mCi/mmol; New England Nuclear, Boston, Mass.). Controls and CD media all contained 0.3% DMSO. The cultures were rinsed twice with cold EBSS, trypsinized, transferred quantitatively to glass scintillation vials, solubilized, and counted as described above.

RESULTS

Descriptive Overview

The changes visible during the first 8 h of exposure to CD in the range from 0.25 to 2.0 μg/ml essentially were manifestations of seven early processes: (*a*) withdrawal of microvilli; (*b*) inhibition of gross movements of cell membrane (ruffling and macropinocytosis); (*c*) loss of translatory cell motility (gliding); (*d*) sustained cytoplasmic contraction; (*e*) herniation of endoplasmic contents including the nucleus; (*f*) aggregation of protrusions at the cell surface; (*g*) eventually, some

[3] Leitz Technical Information Bulletin. 1971. E. Leitz, Inc., Rockleigh, N. J. 11:1–8.

TABLE III

Effect of CD on Adhesivity

	Percentage of cells removed by trypsinization		
	HeLa	Vero	MDBK
DMSO 0.1% 1 h	35	27	25
CD 0.2 μg/ml/1 h	40	34	32
CD 1.0 μg/ml/1 h	27	37	41
DMSO 0.1%/24 h	26	38	32
CD 0.2 μg/ml/24 h	25	45	28

Replicate cultures were planted at subconfluent density (2×10^5 cells) in 35-mm plastic culture dishes. The adherent monolayers were treated with DMSO or CD, as indicated; the DMSO concentration in the CD-containing media was always adjusted to 0.1%. The cell sheets were then trypsinized at 37°C for 10 min (HeLa) or 20 min (Vero and MDBK), and the number of cells detached counted electronically. For detaching the cells, trypsin was diluted with calcium-free saline (Spinner balanced salt solution) to that concentration capable of detaching 25–35% of the cells of a normal monolayer in 10 or 20 min: for HeLa cells this was 0.05%; for Vero, 0.125%; and for MDBK, 0.5% trypsin.

expansion and flattening. These effects were readily reversible on withdrawal of the drug.

Contraction began with 2–3 min of exposure to moderate concentrations of CD (in the range 0.25–0.50 μg/ml). Onset of contraction was accompanied by changes in distribution of microfilaments and the protrusion of blunt, knobby projections of endoplasm (zeiosis) (Fig. 13 *c*). Protrusion of the nucleus usually followed within an hour. While firmly adherent to the substratum, the contracting cells retracted their extended peripheral cytoplasm centripetally, except for the multiple processes that anchored them to the surface or attached them to their neighbors (see Figs. 9, 12, 13). Some 2–6 h later, in the continued presence of CD, the cytoplasm began to spread out in a thin layer and some of the multiple processes were withdrawn. In this interval the zeiotic protuberances had clustered together, and these subsequently moved toward the apex of the free surface, to coalesce into one or two large aggregates ("bouquets"). As with other cytochalasins, cytokinesis of mitotic cells could not be completed in the presence of CD.

Vero and L cells were strongly contracted by CD, and radiated more numerous, narrow, branched, and nodose cytoplasmic processes than HeLa, HEp2, or KB; these cells therefore had a "spidery" appearance. They protruded and even extruded their nuclei more readily than cells of other lines, but responded less with cytoplasmic zeiosis. Except for general contraction, the MDBK line was relatively resistant to the induction of other grossly visible changes by CD.

Comparison of CB and CD

Most of the morphological consequences of exposure to CD could also be procured with CB, but only at higher concentrations (Fig. 1). The maximal contracture attained by HeLa at any concentration of CB was some 40–50% less than the maximum decrease of basal area brought about by CD. Vero were less responsive than HeLa to both congeners up to 3–4 μg/ml, but responded similarly above this range.

Differential Sensitivity of Five Cell Lines to CD

The relative reactivity of the different cell lines to CD was compared with the ultimate intention

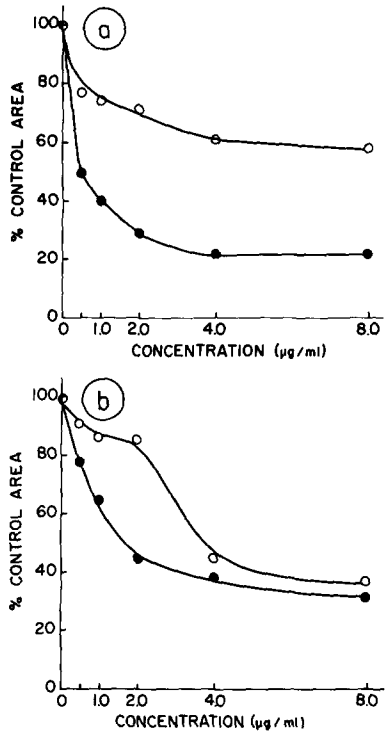


FIGURE 1 Classimat computer analysis of area occupied by cells in subconfluent monolayers after treatment with CB(○) or CD(●). Each point represents the average area of 2,000–4,000 cells, expressed as percent of control area. (*a*) HeLa treated for 2 h; (*b*) Vero treated for 1 h.

of correlating responsiveness with some inherent property. Fig. 2 shows that HeLa and HEp2, the most sensitive cells studied, could contract at minimal concentrations of CD (0.03 μg/ml) which did not measurably affect the other lines, and that doses as low as 0.125 μg/ml in Vero and in HeLa and HEp2 induced 25–30% reduction of area, but only minor contraction of L and MDBK. At doses exceeding 0.2 μg/ml, there was marked retraction and consequent areal reduction of at least 45% in all cell lines at 1 h. Cytoplasmic zeiosis (Fig. 3) occurred much more readily in the epithelial HeLa and HEp2 than in other lines, and was striking in these cells at dose levels (e.g. 0.05 μg/ml) which evoked little or no endoplasmic protrusion in Vero and L. In the MDBK cell line (or in fibroblasts, whether primary or of the collagen-secreting PR 105 strain), there was little or no zeiosis even at the highest concentration of CD.

Nuclear protrusion, although functionally related to zeiosis, gave a somewhat different dose-response pattern (Fig. 4). Monolayers of Vero and L cells were more sensitive to CD than were HeLa or HEp2 cells, while less than 20% of MDBK cells had protuberant nuclei even at 2.0 μg/ml. Once a threshold concentration was exceeded, the majority of competent cells responded precipitately, giving the steep sigmoidal dose-response curves seen in Fig. 4. Extrusion of nuclei usually followed by an interval of time or dose the occurrence of zeiosis, although L cells sometimes did extrude nuclei more or less simultaneously. Comparison of these time sequences and the dose responses suggests that contraction is a prior and necessary event in the extrusive phenomena, but that it is not a sufficient condition, since in cell types like MDBK and fibroblasts, protrusion does not necessarily follow upon cell contraction.

The adhesivity of the established monolayers (Table III) is also correlated with their relative sensitivity to CD. HeLa and HEp2 cells, most easily dislodged by trypsinization, were also most responsive to CD. The MDBK line, notoriously tenacious to the substrate, was also the most resistant to the contractile and zeiotic effects of CD. Vero and L cells were intermediate between these, with respect to both adhesiveness and dose response to CD.

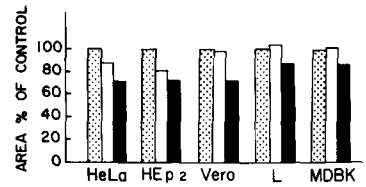


FIGURE 2 Contractile response of different cell lines to low doses of CD. Monolayers of the five cell types at a density near confluence were exposed to 0.01% DMSO in growth medium (control), or to the indicated concentrations of CD for 1 h. Specimens were fixed and stained, and the mean area of 5000 or 1,000 cells was determined by morphometric analysis. The decreased area occupied by the cells is a measure of their contraction. Control (⊡), 0.03 μg/ml CD (□), 0.125 μg/ml CD (■). Standard errors: ± 2.3–4.1%.

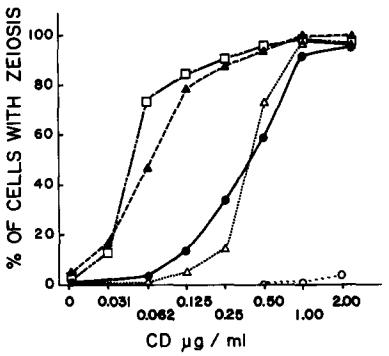


FIGURE 3 Differential sensitivity of five cell lines to induction of zeiosis by CD. The proportion of cells in monolayers showing zeiotic protuberances after exposure to CD for 1 h is plotted as a function of the concentration of CD. 1,000 fixed cells were scored for each point. HeLa (□), HEp2 (▲), L (△), Vero (●), MDBK (○).

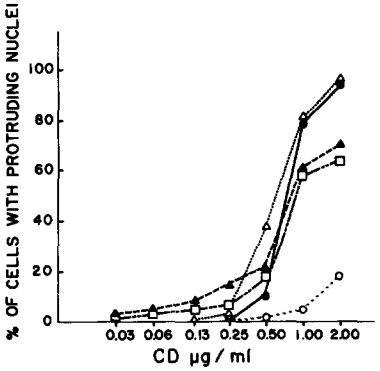


FIGURE 4 Differential capability of five cell lines to express their nuclei on exposure to CD. The proportion of cells in monolayers showing nuclear protrusion is plotted as a function of concentration. 1,000 fixed cells were scored with a phase objective for each point. HeLa (□), HEp2 (▲), L (△), Vero (●), MDBK (○).

Differential Sensitivity among Cells of the Same Line

In the range of concentrations below those yielding maximum effects, the response of a random population of any cell line varied considerably from cell to cell in the degree of visible change brought about by CD, with respect to any of the parameters observed. That this variability reflected differences in responsiveness at different phases of the cell cycle was made apparent in autoradiograms of nonsynchronous cultures of HeLa cells labeled with [^{3}H]TdR and exposed to 0.2 μg/ml of CD (Fig. 5). In these, the proportion of contracted cells with zeiosis was least in the heavily labeled population (mid to late S), and highest in the small, paired, unlabeled cells (putatively, early G_1). More precise information was obtained from synchronized cultures initiated by cells in mitosis dislodged from HeLa monolayers by mechanical agitation. Some cultures were allowed to proceed into G_1; others were arrested at the G_1-S interphase with 7.5 mM thymidine, upon relief of which block the cells proceeded nearly synchronously through S and into G_2. The synchronized cells were exposed simultaneously to [^{3}H]TdR and CD at intervals suitable to identify the response at each phase of the cell cycle. The results (Fig. 6) showed that G_1 was most sensitive to the effects of CD, and that in early S responsiveness began to decline and was lowest by mid S, at which level it remained through most of G_2. These differences became less distinct at higher concentrations, and were virtually obliterated in HeLa above 0.8 μg/ml of CD.

Cell Contraction

Cells of every line examined (including also chick fibroblasts and PR 105 fibroblasts) responded rapidly to CD by contracting. This response was sustained in at least a part of the cytoplasm for as long as it was exposed to CD, hence the term contracture (sustained or prolonged tonic contraction) is applicable. Withdrawal of extended ectoplasm and the heaping of cytoplasm at the

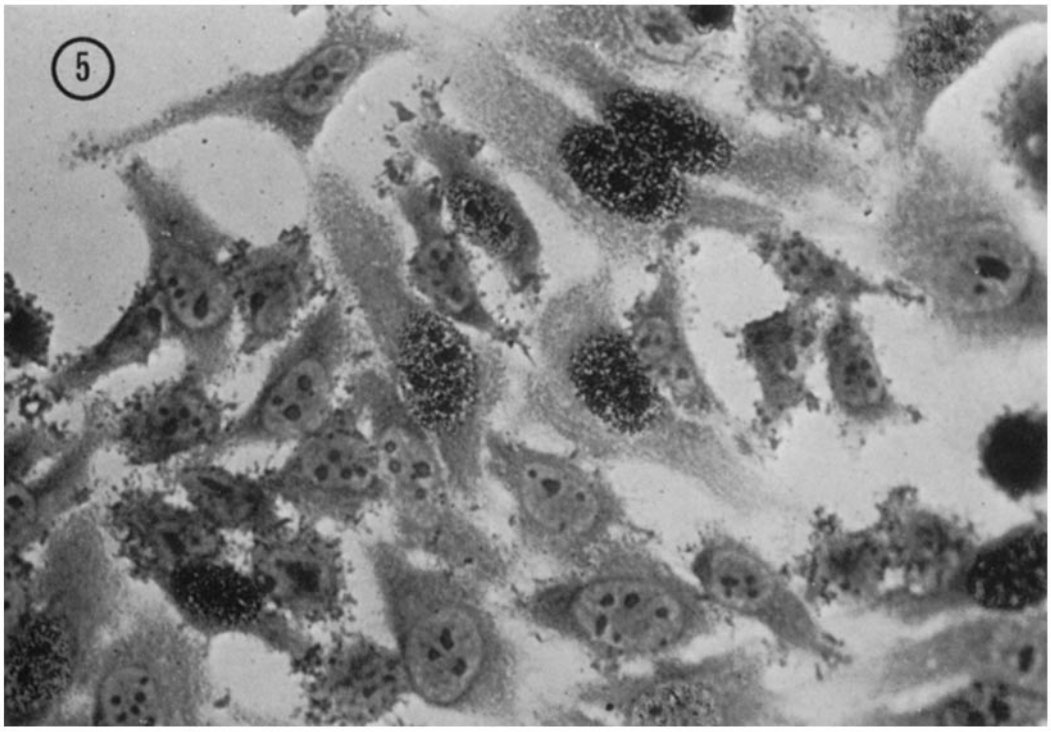


FIGURE 5 Autoradiograph of HeLa cell culture treated simultaneously with 0.2 μg/ml of CD and 10 μCi/ml of tritiated thymidine for 10 min. Most of the heavily labeled cells (S phase) do not exhibit any contracture or zeiosis at this time, while unlabeled cells show marked CD-induced effects. × 500.

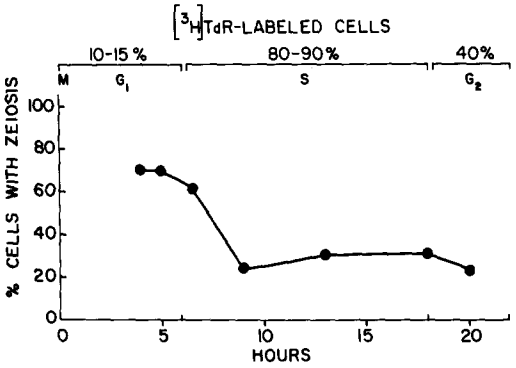


FIGURE 6 HeLa cells were synchronized by selective detachment of mitotic cells which were allowed to proceed to G_1. Some of these were then exposed to a thymidine blockade procedure, as described in Materials and Methods, to obtain nearly synchronous populations of cells in S and G_2. The abscissa refers to number of hours after mitosis (0 h) as extrapolated from the labeling data. The synchronized HeLa cells were treated simultaneously with 0.2 μg/ml of CD and 10 μCi/ml of tritiated thymidine for 10 min, the latter to determine the proportion of labeled cells at each interval. The percentage of cells exhibiting zeiosis was scored and corrected for the small number of cells with zeiosis in control populations (G_1 < 5%; S and G_2 < 1%).

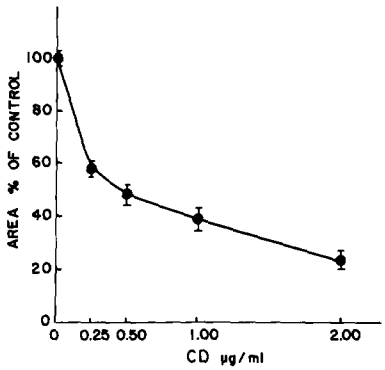


FIGURE 7 Contractile response of MDBK cells as a function of dose. Monolayers were treated with the indicated concentrations of CD for 5 h. The mean area of 500 cells in each sample was measured morphometrically; standard errors are given.

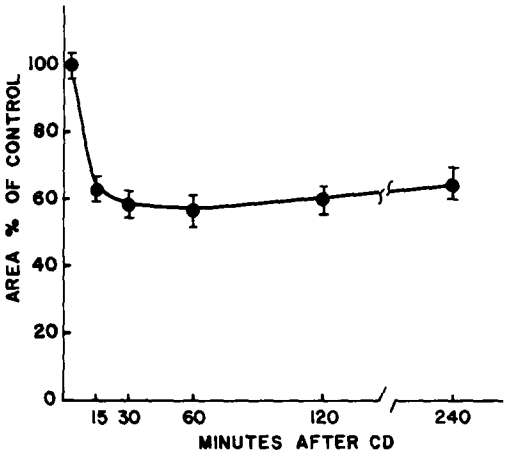


FIGURE 8 Contractile response of HeLa cells as a function of time. The area occupied by HeLa cells in monolayer at successive intervals of exposure to 0.25 μg/ml of CD, was measured morphometrically in fixed preparations; each point represents the mean area of 600 cells; standard errors are indicated.

apex of the cell was accompanied by shortening (retraction) in all planar dimensions and increased convexity. As a result the area covered by the cell body diminished in proportion as the cell was contracted, and this areal diminution has been measured as an index of the degree of contraction. Using this parameter, contraction was found to be the first and most sensitive of the visible responses; and its extent was dose dependent within a limiting range of concentrations (Figs. 1 and 7). The major contractile response occurred precipitately once the threshold concentrations were exceeded, and continued thereafter by smaller steps up to a limit of about 2 μg/ml (Figs. 1 and 7). In HeLa, HEp2, or Vero, maximum contracture was achieved within 30–40 min at median concentrations (Fig. 8), and the cell area could be reduced to as little as one-fourth or less of its initial value (Figs. 9 and 10).

At maximum contraction there was a minor but consistent loss of volume of about 10–15% which was promptly regained during recovery after withdrawal of the drug (Fig. 11).

The contracture was chiefly cortical, and involved the broad extended sheets of ectoplasm as well as the cell processes. Constriction of the latter was clearly evident as part of the general contraction. Those processes anchored to other cells or to substrate did not detach in the initial wave of contraction; they appeared much longer, many of them as though stretched and pulled taut as the distance increased between their sites of adhesion and the main body of the cell of origin (Figs. 9, 10, 12, 13).

Model Systems

The glycerol-extracted muscle fiber has long served as an instructive model system for the study of contractility, a property shared by most, if not

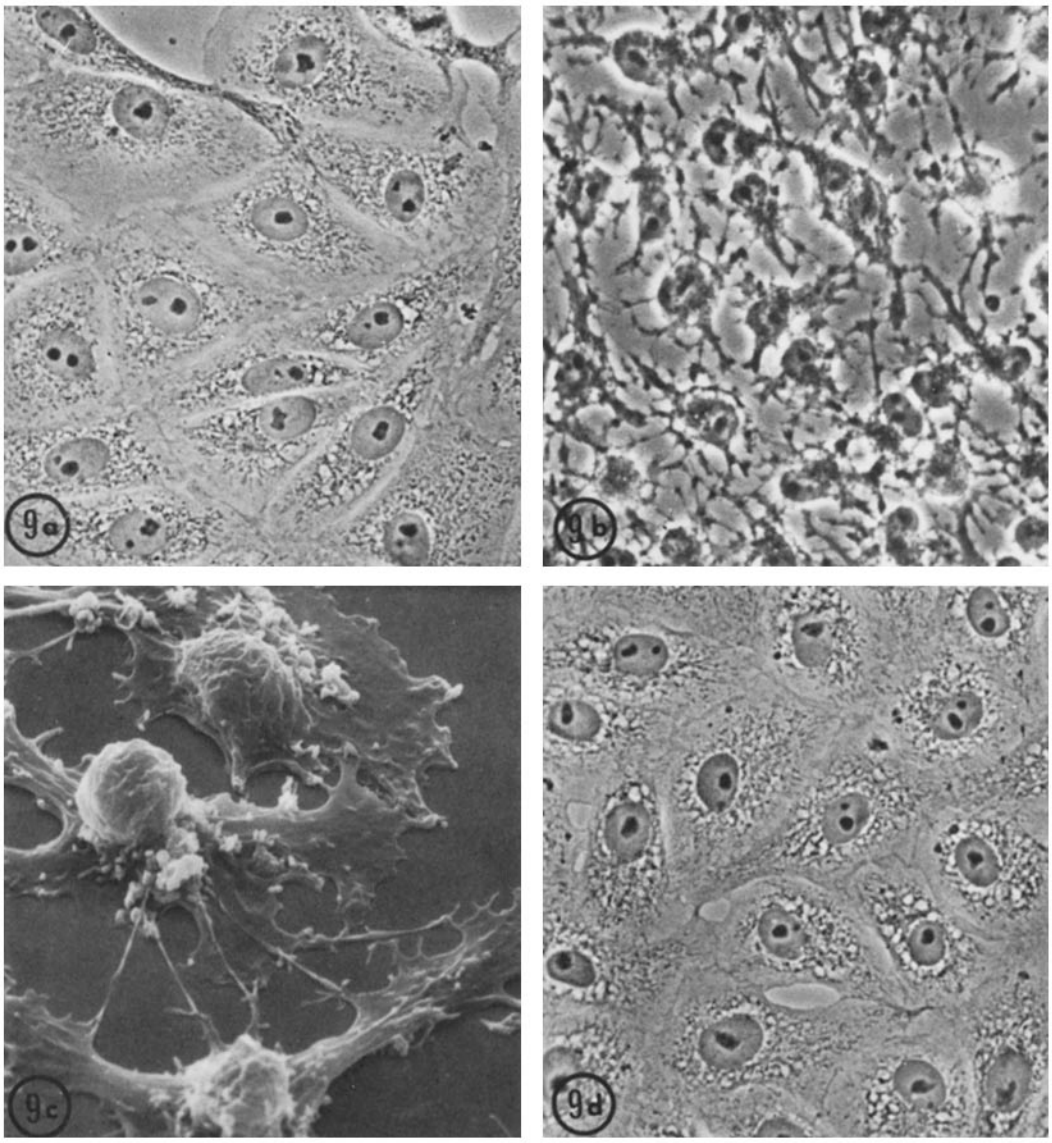


FIGURE 9 (*a*) Vero cells in normal growth medium with 0.2% DMSO, showing contiguous margins of flattened cells. The thin velum of attenuated ectoplasm is especially evident at the free margin of the cell at upper center; a mitotic figure is at the upper right. Fixed preparation, phase contrast. × 360. (*b*) A similar monolayer of Vero cells 1 h after application of 2 μg/ml CD. The cells are maximally contracted, all the nuclei protrude into the upper focal plane, and branched and contracted processes radiate from the cells. Fixed preparation, phase contrast. × 360. (*c*) Vero cells, similar to those in (*b*), 1 h after exposure to 2 μg/ml CD as visualized in the scanning electron microscope. Macro- and microprocesses, in contact with adjacent cells extend from the generally contracted cell bodies, from which nuclei and small clusters of zeiotic knobs protrude. × 1,600. (*d*) A similar field of Vero 1 h after refeeding with fresh drug-free medium. Restitution to normal appearance is nearly complete. Fixed preparation, phase contrast. × 360.

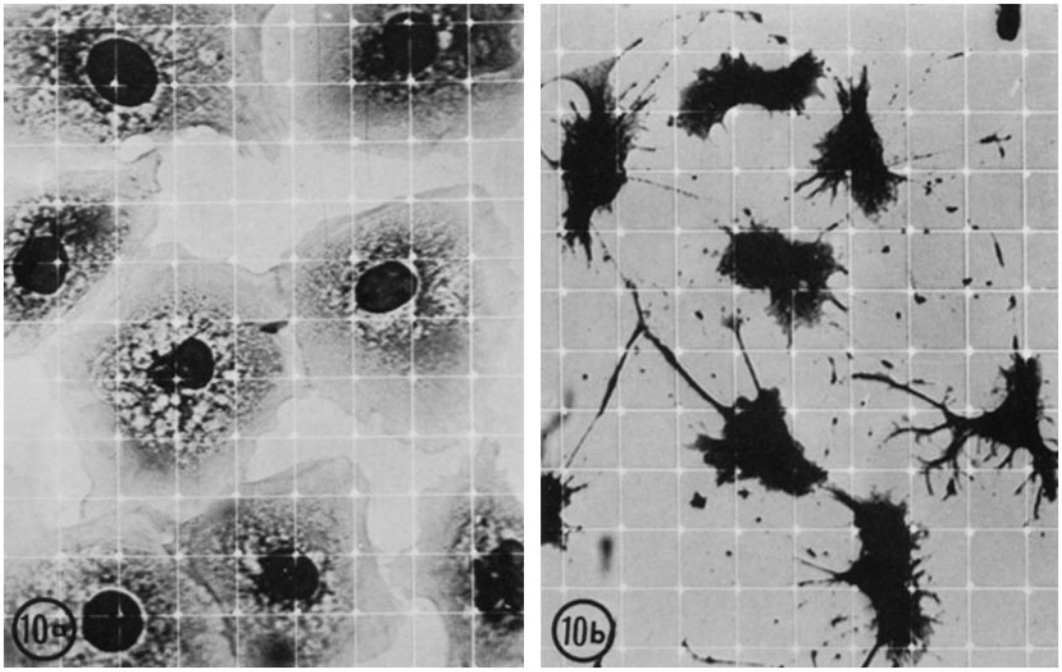


FIGURE 10 MDBK cells in monolayer stained with haematoxylin and crystal violet to show cell margins, as they appear in 0.2% DMSO control medium (*a*), and at 5 h after treatment with 2 μg/ml CD (*b*). The photographs used in morphometric determination of area were overlaid during printing with a grid of 1 cm^2. The degree of CD-induced contracture is recorded in Fig. 7. The straight, nodose, cytoplasmic processes may extend away from the contracted cell body for distances exceeding 40 μm. × 560.

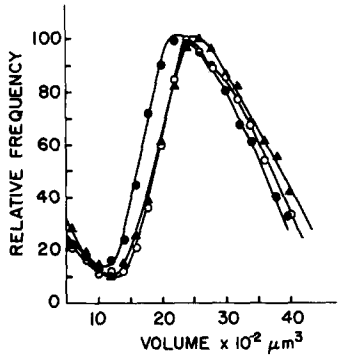
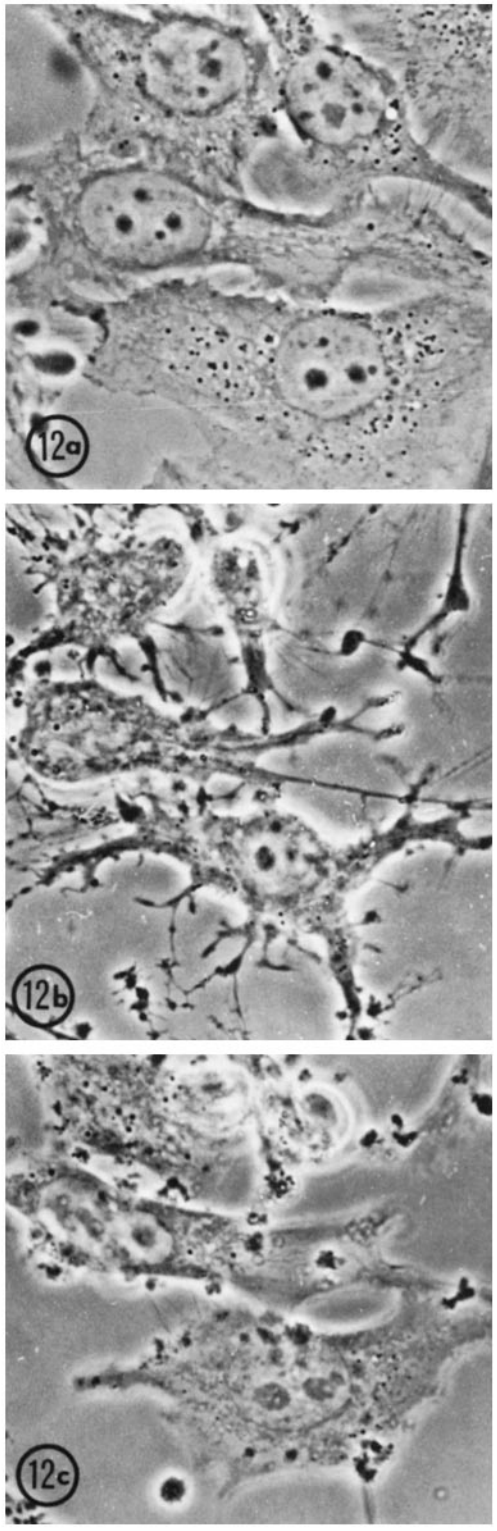


FIGURE 11 Reversible CD-induced volume changes in HeLa S-3 suspension cultures. Relative frequencies of volume classes obtained from Coulter counts were normalized to permit comparison of curve shape. DMSO control (○); 1 μg/ml CD, 3 h (●); 1 μg/ml CD, 2 h, refed with drug-free medium, 2 h (▲).

all glycerated cells (43, 98). The structures participating in this movement remain intact and require only ATP (and Ca^{++}) to effect contraction and shortening. There is little doubt that the basic mechanisms, the utilization of ATP, and the transfer of energy during the phase of contraction are fundamentally the same in all such models, and presumably also in intact cells.

CD itself, in concentration from 0.25 to 0.5 μg/ml, caused little or no perceptible change in glycerinated models of HeLa or Vero cells. Neither did CD prevent the well-marked contraction of such models induced by 5×10^{-3} M ATP, whether they were pretreated with 0.25–0.5 μg/ml CD during the 5–6-wk period of glycerination, or when CD was applied 15 min before and during the application of ATP, although a slight diminution of the extent of contraction of these models was observed. Since the contractile apparatus was capable of shortening after glycerination, these results would suggest that in its action on the living cell, CD requires the presence of substance(s) removed during glycerol extraction, presumably ATP, and also that CD does not directly affect the functional integrity, that is the ability to contract, of the basic actomyosin-like structures themselves.

That cell contraction caused by CD is as de-



pendent on the continued metabolic provision of intracellular sources of energy (ATP) for chemomechanical transduction as contraction induced by any other means is indicated by the observations that CD-induced contraction was prevented by some agents that inhibit energy metabolism (Table I). DNP and DOG were effective inhibitors of contraction (Fig. 14). However, IAA, cyanide, or azide failed to antagonize cell contraction caused by CD. The inhibition to CD-induced contraction was promptly relieved on withdrawal of the DNP and DOG. These results sustain the impression that cytochalasin does not act either by disorganizing the contractile machinery or by interfering with its source of energy. They further indicate that the contracture induced by cytochalasin in the living cell is an active, energy-dependent process.

Surface Structures and Cell Extensions

Microvilli of HeLa cells were withdrawn within 2–4 min after moderate concentrations of CD, simultaneously with the onset of contracture and the first appearance of zeiotic knobs (Fig. 13 *a*, *b*). With the further development of zeiosis and nuclear protrusion the cell surface became progressively smoother everywhere and residual microvilli were represented by a stubble (Figs. 9 *c*, 13 *c*).

Microextensions (filopodia) were normally prominent in HeLa or Vero only in rounded mitotic cells (Fig. 13 *d*), originating from the subapical convex side of the cell (not at the margin, as in interphase) and often terminating in regularly

FIGURE 12 (*a*) Four living HeLa cells 2.5 min after addition of medium containing 2 μg/ml CD. Slight to moderate retraction of the cytoplasm has already occurred. (*b*) The same field 4 h later. All cells have contracted markedly, leaving long, straight, often branched processes in contact with neighboring cells or with the substratum. Dense cytoplasmic protuberances are evident all about the periphery and on some processes. The nuclei have been displaced eccentrically; in the upper three cells they protrude above the focal plane and are represented by phase halos. (*c*) The same field 40 min after withdrawal of CD and refeeding with fresh drug-free medium, shows partial restitution. The cells have recovered most of their original area. The nucleus of the lowest cell is replaced and flattened; the nuclei of the upper cells are in the process of reinsertion. Dense cytoplasmic protuberances are still evident at the cell peripheries. Phase contrast. × 920.

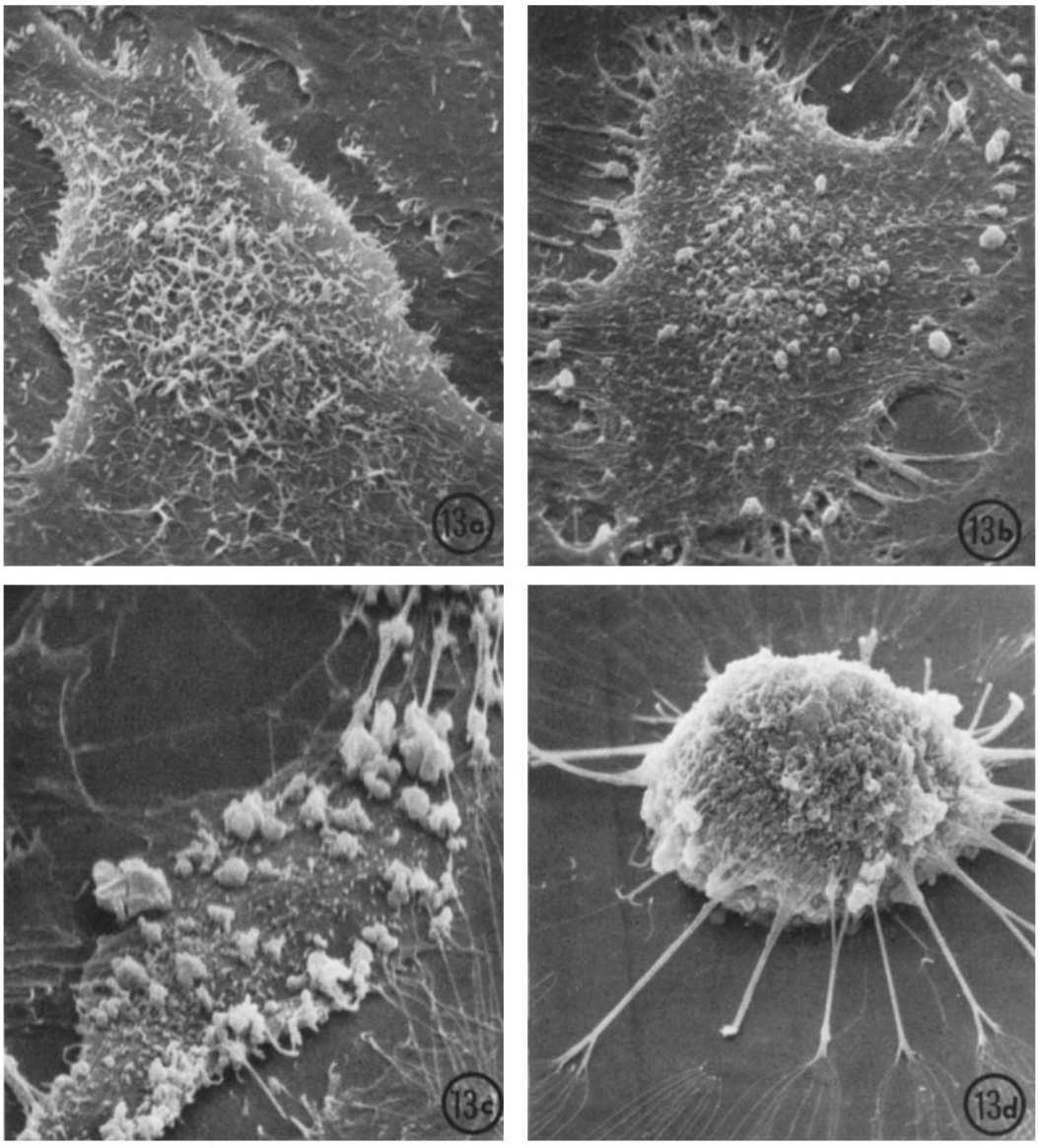


FIGURE 13 (*a*) Surface of an extended HeLa cell in monolayer, grown in 0.025% DMSO in normal medium, as seen in the scanning electron microscope. Microvilli, present on most of the free surfaces, are most abundant over the nucleated center, and least in evidence over the extended ectoplasm of the margins. Not all the HeLa cells have as many microvilli. (*b*) A HeLa cell, 3 min after exposure to 0.25 μg/ml CD, which has begun to retract. Numerous microprocesses are evident at the periphery. The microvilli have been, or are in course of being withdrawn and a number of small zeiotic protuberances are present over the cell center, at the margins, and on some processes. (*c*) On the surface of a contracted HeLa cell, 45 min after exposure to 0.25 μg/ml CD, the microvilli are represented only by a stubble. Many rather large zeiotic knobs beset the free surface, especially at the margins where they are clustered. Contact with adjacent cells is maintained by long filopodia. Stouter retraction fibers are at upper right. (*d*) A HeLa cell in early mitosis at 4 h of exposure to 0.25 μg/ml CD. The surface is finely corrugated and a few zeiotic knobs are present, especially at the convex margin. The cell is guyed by some 20 taut processes radiating from the edge of the convex slope. They are attached to the glass surface by means of fine filar pennae splayed out from their extremities. × 2,000.

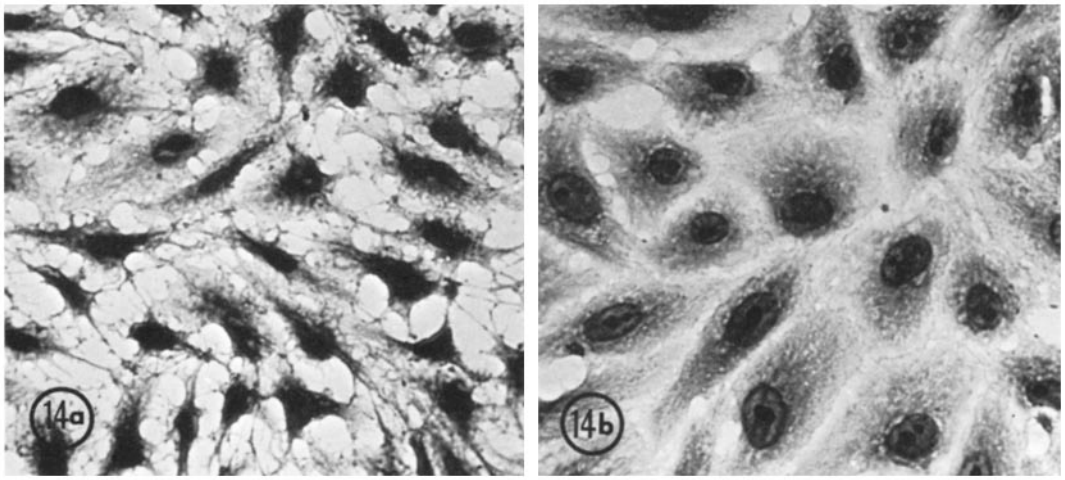


FIGURE 14 (*a*) Vero cells strongly contracted by application of 1 μg/ml CD for 15 min. Monolayer fixed in neutral Formalin and stained with haematoxylin and crystal violet to reveal cell borders (*b*). A similar monolayer of Vero preincubated in 5×10^{-3} M DNP and exposed to 1 μg/ml of CD and 5×10^{-3} M DNP for 15 min. The contractile response has been prevented and the cells remain in their fully extended condition. × 315.

splayed out filar attachments (Fig. 13 *d*). On exposure to CD, very numerous filopodia and subsequently larger processes became evident as the ectoplasm contracted (Fig. 13 *b*, *c*). They remained adherent to the substrate or to adjacent cells (Fig. 13 *c*), and became longer and straighter (taut) as contracture progressed. Most continued to adhere even when retraction was near maximal (Figs. 9 *c*, 12 *b*, 13 *c*); a few, however, snapped and recoiled upon the cell body, forming tabs almost indistinguishable from small zeiotic knobs.

Cells remained in mutual contact both through the lengthened filar microextensions terminating on adjacent cells, and along the broader sectors of wide processes (Fig. 9 *c*). At all these zones of intercellular adherence, the closely approximated membranes were not more than 20 nm apart. Within these areas of apposition, there were foci or points of closer contiguity as in untreated cells (Fig. 15). These various specialized points of closest apposition, as visualized in the transmission electron microscope, were interpreted as probably "intermediate" (45) and/or gap junctions.

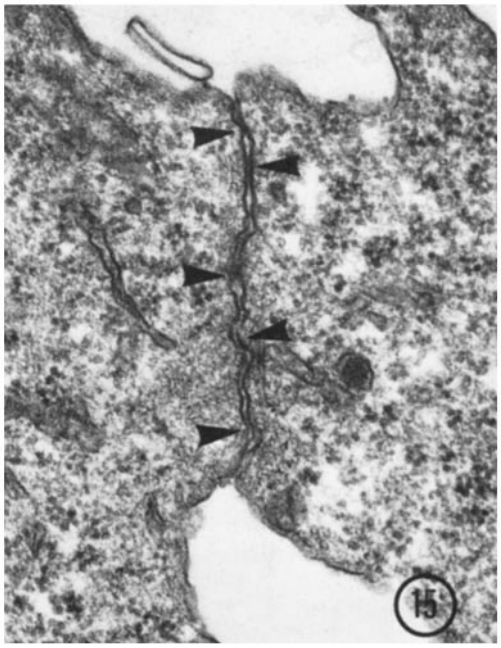


FIGURE 15 Appositional sector of mutually contiguous retracting HeLa cells, after 1 h of exposure to 0.25 μg/ml CD, visualized in the transmission electron microscope. Their membranes are separated by a space of 10–18 nm. At focal points (arrowheads) the outer membrane leaflets are still in close approximation, separated by a space of not more than 3 nm. These may be specialized junctions. × 50,000.

Attachment and Adhesivity

The data of Table II show that concentrations of CD capable of bringing about contracture and change of cell shape also inhibited (but did not abolish) the processes of settling and attachment of cells to substrate, i.e., fewer cells became adher-

ent to the glass. In the presence of CD, the rounded cells failed to spread on the surface, nor did they extend the usual cytoplasmic processes. As indicated in Figs. 2 and 3, MDBK was less affected by CD than the other cell types with respect to contraction and zeiosis, and this appears also to be true of attachment. Although 0.25 μg/ml of CD caused contraction of these cells (Fig. 7), this dose did not appear to have interfered with their ability to settle and adhere to surfaces. Only at higher concentrations, e.g. 1.0 μg/ml, did MDBK show a significant impairment of attachability.

The influence of CD on the detachability of established monolayers adherent to plastic surfaces is given in Table III. Notwithstanding pronounced retraction and morphological change effected by 0.2 μg/ml of CD for 1–2 h (Figs. 1 and 8), the three cell types studied were not more easily detachable by trypsin; on the contrary, the adhesivity of HeLa would appear to have been somewhat enhanced. It is also remarkable that prolonged exposure to CD (e.g. for 24 h), during which a proportion of the cells had begun to flatten and resume a more discoid shape, had no significant effect on their adhesivity, i.e., detachability by trypsin (Table III).

Permeability to DOG

Decrease, most often to complete abolition of permeability to various hexoses is reportedly one of the earliest consequences of exposure to CB in several cell types (20, 23, 32, 51, 62, 115), and can apparently occur at dose levels as low as 0.003 μg/ml of CB. The uptake of DOG, which penetrates by the same kind of transport mechanism as glucose, but is not metabolized, is a convenient indicator of the permeability of the cell membrane to hexoses. CD ultimately curtailed the entry of [^{14}C]-DOG into HeLa by 40–50%; this inhibition began abruptly at 0.003 μg/ml, whereafter even high concentrations of CD effected little further change in permeability. The permeability of MDBK and Vero cells to DOG on the other hand was not significantly affected at any nontoxic concentration of CD (Table IV).

The minimal concentration of CD effective in reducing permeability of HeLa to DOG was thus lower by about an order of magnitude than that required to effect a recognizable morphological change in these cells. Vero, although insensitive to CD with respect to sugar uptake, responded readily with contraction and simplification of its surface at concentrations above 0.03 μg/ml, and MDBK, although relatively more resistant, nevertheless contracted to some 40–50% of its normal area at 0.25 μg/ml (Fig. 7).

There was thus no correlation between the occurrence either of early changes at the cell surface or subsequent contracture and the loss of permeability to DOG. These would appear to be independent events.

TABLE IV

Effect of CD on Permeability to DOG

CD	Percent inhibition		
	HeLa	Vero	MDBK
μg/ml			
0.001	0	0	0
0.003	37	—	—
0.01	44	5	0
0.03	44	—	0
0.30	44	—	0
1.0	44	—	—
1.5	47	10	0

Uptake of 2-deoxy-D-[^{14}C]glucose was monitored as described in Materials and Methods. HeLa and MDBK cells were incubated with CD and DOG for 30 min; Vero for 15 min.

DISCUSSION

Cytochalasin begins to act immediately and the changes it induces go to completion simultaneously with cytoplasmic contracture. Sustained contraction of the whole cytoplasmic mass is the dominant feature of the first 4–6 h of exposure to CD in all cell lines. Herniation of endoplasmic contents through the cortex occurring *pari passu* with contraction, achieves dramatic proportions after attainment of maximal contracture (Figs. 9 *b*, *c*, 13 *b*, *c*). This is a form of zeiosis in which endoplasm and then nucleus are protruded through the cytoplasmic cortex to bulge at the free surface and is only partly the result of increased intracellular pressure generated during contracture; it will be illustrated and discussed in an ensuring communication.[1]

Differential Sensitivity

Virtually all types of animal cells appear to be affected by cytochalasin. In comparing the responsiveness of several lines, it was hoped that differences in reactivity and sensitivity could be correlated with other significant parameters, such as

content and arrangement of filaments, nucleotide content, or extent of binding of radioactive cytochalasin (55, 93), to afford some clues about the mechanism of action of the cytochalasins. The reasons for differences in sensitivity to CD between different cell lines have not yet been resolved on any of these bases. But the ultimate explanation for the equally arresting differences in responsiveness from cell to cell within a nonsynchronous population of a given cell line may prove applicable to differences between cell types. The data show (Figs. 5 and 6) that the differences in sensitivity to cytochalasin among cells of the same type are attributable to phase differences in their progression through the cell cycle, cells in G_1 being much more responsive than cells in S and G_2. Whether this can be correlated, in turn, with a specific parameter such as fluctuation in cyclic nucleotide content (13, 82, 113) remains to be demonstrated, but the evidence suggests an inverse correlation between responsiveness to CD and intracellular level of cyclic AMP. The inverse correlation between adhesiveness of the various cell types to substrate and their apparent responsiveness to CD may also be related to the differential responsiveness of individual cells. It is pertinent that adhesivity of monolayers is greatly increased by the addition of dibutyryl cyclic AMP (44) and their mobility is correspondingly diminished (46). Prior application of dibutyryl cyclic AMP, under appropriate conditions, markedly diminishes the sensitivity of cell lines such as HeLa to the contractile (60) and zeiotic effects of CD (60, 75). Taken together, these facts suggest, as a hypothesis to be evaluated, that intracellular levels of cyclic nucleotide may govern the responsivity of cells to CD and and their contraction. These levels can be expected to differ in different cell lines, as they do in different phases of the cell cycle.

Permeability

Unanticipated from any previously known action of cytochalasin was the discovery that permeability of some cell types of hexoses (glucose, deoxyglucose, methylglucose, glucosamine, galactose) is immediately inhibited by CB (20, 23, 32, 51, 62, 115). Fibroblasts, hepatoma and Chang liver cells, L cells, and adipose cells in vitro are among those in which transport of hexose is drastically but reversibly diminished by CB, an effect which can become manifest in cultured chick fibroblasts at concentrations as low as 0.01 μg/ml (50). This inhibition is fairly selective; nucleoside is also excluded from hepatoma cells but not from most other cell types in the presence of CB (62, 68), and transport of other small molecules such as choline, orotate, aminoisobutyrate, or amino acids is not significantly changed (20, 32, 62, 68, 115). Kinetic analyses, based chiefly on Lineweaver-Burke plots, offer contradictory interpretations of the nature of inhibition in different cell types. In chick fibroblasts and in Novikoff hepatoma cells, CB acts as an inhibitor competitive with these sugars for membrane transport sites (32, 50); while in leukocytes, L cells, HeLa, and 3T3 cells the inhibition of transport is said to be noncompetitive (62, 115), as it also appears to be in fat cells (23). It is agreed that phosphorylation of hexose is not disturbed by cytochalasin (23, 32, 50, 62). Neither cell membrane ATPase activity, nor the cellular contents of ATP or cyclic AMP, are reportedly influenced by CB at least up to 4 h of exposure (50, 97).

Entry of hexose into HeLa can be suppressed only by about 50% even at high concentrations of CB (62, 92) or CD (Table IV). Whether cytochalasins are competitive, or more probably, noncompetitive inhibitors of hexose transport sites at the HeLa membrane, their inability to reduce uptake by more than 50% would suggest that there may be cytochalasin-insensitive transport sites, or (less probably) that entry of glucose in about half of a random population of HeLa cells is refractory to inhibition by cytochalasin.

Doses of CB that inhibit hexose uptake of fibroblasts by about 50% are insufficient by at least an order of magnitude to effect changes in cell shape (50). Conversely, CA, notwithstanding its rapid effect on cell form, is relatively ineffective in blocking sugar uptake in some of the same cell types (51). CD only partly inhibits uptake of DOG into HeLa, and has virtually no influence on the transport of this sugar into Vero, MDBK or HEp2 cells at any nontoxic dose (Table IV), although permeability of all these cell lines is definitely inhibited by CB.[2] Yet CD is much more potent in bringing about marked contractile and morphological changes in these lines. Thus, the marked differences in CB concentration affecting permeability in susceptible cells and those producing visible changes, the dissociation of effects on transport at the cell membrane, and the capacity to effect morphological changes among the different cytochalasins (A, B, and D), and in different cell lines, reinforce the opinion (50, 51) that the mechanisms

by which cytochalasins affect sugar transport are causally unrelated to its other actions.

Cell Surface Structures

It has sometimes been theorized that microvilli have an important part in transport functions, if only by extending available surface. Thus, the villous brush borders of absorptive cells have been thought to take part in the active transport of sugars, and a correlation has also been made between the high rate of glucose transport of transformed cells and their more numerous microvilli (10, 71), and between the ambient glucose concentration and the luxuriance of microvilli of HeLa (107). In our experiments no necessary relationship could be discerned between the inhibition (or noninhibition) of hexose uptake by CD, and the form and number of microvilli. Loss of microvilli, which occurs in all types of cells under the influence of CD with the earliest onset of contraction, is not accompanied by diminished permeability to DOG in Vero and MDBK. Withdrawal of microvilli by these cells need not represent a net change of surface area, a parameter perhaps more relevant to transport.

The long microextensions of mitotic or trypsinized cells have been interpreted as retraction fibrils "spun out" from the retreating border of the cell as it rounds up and pulls in from its flattened condition (24, 25, 90). This rather simplistic formulation can also be invoked for many of the processes of the cell retracting under the influence of CD. It is assumed that each long microprocess is anchored to the substratum at sites of adhesion formed during the extended state of the cell, and which have held fast during CD-induced retraction of the main cell body and much of its extended ectoplasmic margin.

Cell Attachment and Adhesivity

A distinction has been drawn in our experiments between the processes that effect the contact, settling, spreading, and attachment of suspended cells to electronegative surfaces (such as serum-coated glass), and the adhesiveness or stickiness of flattened cells which have already established themselves as monolayer on a substrate. The relation of mechanisms of attachment or adhesivity (101) to contractility and movement, are not completely understood. We have found that CD inhibits settling and attachment of several cell lines, but has little effect on the stickiness or detachability (adhesivity) of adherent cells, and may indeed even enhance it somewhat. Inhibition of settling and attachment of cells by CB has been reported by Weiss (102) and Goldman (37), who found colchicine to be without effect on this process. Our observations accord with the interpretation of these investigators and of others (109) that active protrusion of filopodia or microprocesses, as well as the capacity of the ectoplasm to spread by the extension of an undulating velum, is essential to attachment, and these processes are compromised by cytochalasin. The attachment of cells has been postulated, on the basis of other evidence, to entail a contractile event (48) involving expansion of the cell surface (47).

Maximally contracted cells in the presence of CD continue to adhere firmly to substrate (Table III). While at first estimation it might seem that adhesivity could be correlated with basal cell surface area approximated to substrate, our data (Figs. 7–10 and Table III) show that this is clearly not the case. Most of the basal or underside of the cell is said to be separated by 20 nm or more from the surface, to which it is weakly adherent, probably by secondary attractive forces (22, 100). Cells are firmly anchored to substratum only at limited, circumscribed foci, most probably by special maculate attachment devices (described as "pillar-like" [12]) which approach to within 10 nm or less of the substrate surface (2, 12, 77). Attachment also occurs at fine peripheral attachment bridges (21), and by means of the microextensions (24, 25, 71). The tenacity with which cells adhere in the presence of CD, notwithstanding drastic reduction of overall surface area, is best explained by assuming that the special attachment points remain intact, and that cytoplasmic retraction affects only the wide intervening areas between the special sites of adherence. The long, branching microextensions that radiate from the contracted cell body, causing the "spidery" appearance of many cell types exposed to cytochalasin (78), may be regarded as "retraction fibers" which terminate in, and/or bear special attachment devices along their undersides. Cells adhering by about as many such intact devices in the presence of moderate concentrations of CD as in its absence, would not be expected to detach more easily. Only at high doses of CD, when the rapidity and violence of the cell retraction or the onset of minor toxic effects (e.g. sublethal autolysis [73, 99]) may have caused rupture

of the attachment-maculae and raveling up of microextensions, does it become significantly easier to dislodge cells from substrate.

Contraction

Generalized cytoplasmic retraction incident to exposure to CB has previously been noted only briefly or casually, usually either as a transient (17) or a relatively late event (86, 104). The change in shape effected by this agent in some cell types, giving rise to multiprocessed cells of a remarkable dendriform or arborized appearance has been depicted by other observers (6, 61, 78). The importance of the strong, generalized contraction of ectoplasm as an almost immediate consequence of the action of cytochalasin, seems not to have been appreciated hitherto. The recoil of the marginal cytoplasm under the influence of CD does not follow upon a dissolution of the attachment sites of cells to the substrate, such as may occur during locomotion (16), in mitosis, in the absence of Ca^{++}, or after trypsinization. Contraction after exposure to CD is rather a primary event; it occurs in suspended cells, and does not affect the adhesion of cells already attached. The contracture of L and HeLa cells entails a minor, but definite, loss of volume (Fig. 11) reminiscent of the small reduction in volume during development of tension in muscle (1).

Contractility, a property inherent in all cells, is thought to be effected by an apparatus constructed basically of actin and myosin (or myosin-like) components. These proteins are of general, probably universal, distribution in animal cells (3—5, 69, 70, 84, 112). Although the amounts, and degree of organization of these proteins, and their association with regulatory components is of a much higher order in cells specialized for contraction, it can be assumed that basic mechanisms of contractility are similar in all cells (4, 52, 69, 112). The finding of tropomyosin-like protein in nonmuscle cells (19, 84, 112) makes credible the applicability of a scheme similar to that accepted for muscle to cytoplasmic contraction in general (4, 112). However, other more primitive modalities of regulation of the interaction of actin and myosin than the troponin-tropomyosin system (e.g. phosphorylation) may be more important in nonmuscle cells (3, 5).

Actin filaments, in arrays or in nets, are common to the ectoplasm of all animal cells; organized myosin filaments are not usually present in nonmuscle cells. In these cells, the myosin is probably dispersed and may perform its function in the form of oligomeric aggregates (52, 69). The force developed by the concerted movement of actin microfibrils on myosin is transmitted, in nonmuscle cells, probably chiefly to plasma membrane, onto the inner face of which the microfilaments are inserted by as yet undefined means.

The process in the sequence of contraction and relaxation on which CD exerts a primary action causing a state of generalized tonic contraction is unknown. Among the possible hypotheses are the following: (*a*) an action at the plasma membrane affecting its permeability and/or electrotonic properties; (*b*) an effect on the flux of Ca^{++}, such as to increase Ca^{++} concentration in the cortex; (*c*) an action on the properties of the contractile proteins themselves, or on their regulatory components; (*d*) some effect on the structural integrity of the contractile apparatus, or its attachment sites.

The diminution by some cytochalasins of the permeability of plasma membranes of some cell types for hexoses or nucleosides, the reported failure of membrane fusion in the presence of CB during cytokinesis (9), and the observation that high concentrations of CB can alter the membrane potentials of heart muscle cells in culture (54) lend some credence to the first of these hypotheses. It remains to be validated by electrophysiologic means.

Local intracytoplasmic changes in the free Ca^{++} level affecting contractility may be controlled by the energy-dependent activity of plasma membrane as well as elements of the smooth endoplasmic reticulum (SER) (34, 94). However, the flux of $^{45}Ca^{++}$ across the plasma membrane is reportedly not affected by doses of cytochalasin that inhibit the sugar-transport sites (62). This datum, even if validated by use of the lanthanum technique for studying Ca^{++} exchange (94) would not conclusively rule out the possibility that CD may act somehow to increase the local ectoplasmic concentration of free Ca^{++} by affecting its release from or sequestration in the microsomal (SER) Ca pumps. An important regulator of Ca-binding activity by microsomes (in the presence of ATP) is cyclic AMP, the increase of which promotes Ca sequestration, lowers free Ca, and induces smooth muscle relaxation (7).

Another instance in which phosphorylation of a regulatory protein via cyclic AMP, namely the inhibitor component of troponin (TNI), may govern contractility has been reported (91). A light-chain

component of myosin itself can be phosphorylated (66). The regulatory function of such phosphorylations is probably much more important in nonmuscle cells (3, 5).

Effects of cytochalasins on the basic contractile proteins themselves have been reported. Although reduction of the Mg^{++}-dependent ATPase activity of actomyosin by CB has been reported (64); this has not been found to occur with CD in concentrations up to 2 μg/ml (76). These concentrations of CD depress myosin ATPase activity reversibly, and higher concentrations interfere with the reassociation of actin and myosin (76). Moreover, [^{3}H]CD is said to bind to purified myosin, but not to actin (76).

The integrity of purified F-actin filaments in vitro has been reported to be altered by direct interaction with CB (87, 88); effects, however, denied by Forer et al. (33). A direct disruptive effect of cytochalasin on the microfilaments of the cell cortex (or of their insertions), on a generalized scale such as that tentatively suggested by Wessels et al. (103), and by the findings of Spudich (87), and often repeated in the relevant literature, would hardly seem consonant with the prolonged maintenance of contractile tension such as occurs in cells under the influence of CD. The ultrastructural appearance of the cell cortex, to be described in the ensuing paper,[1] permits of alternative interpretations (see also reference 36). Moreover, neither myofibrillar organization nor the integrity of myofilaments in developing muscle is affected by CD (61). Even if the findings with purified F-actin (87) were transposable to the living cell, this is probably not the primary or principal mode of action of CD.

If the contractile apparatus were affected by CD in such wise as to permit contractile shortening, but to interdict relaxation, i.e., to cause a locking-in of the contracted system, a condition of generalized unremittent contracture, such as is in fact observed, would ensue. This must be considered among the possibilities that remain to be verified.

The speed with which cytochalasin proceeds to act, the observation that the only important cellular binding sites of [^{3}H]CD are plasma and (secondarily) microsomal membrane (93); and the evident hydrophobic-lipophilic character of part of the cytochalasin molecule, are data that tend to favor some constituent(s) of plasma membrane and/or cellular endomembrane as primary sites on which cytochalasin impinges, and the theory that other observable effects such as contraction somehow follow from this interaction.

Addendum

Two more recent publications (Yamada, K., and N. Wessells. 1973. *Dev. Biol.* **31**:413., and Taylor, E., and N. Wessells. 1973. *Dev. Biol.* **31**:421.), provide additional evidence that interference with hexose transport by CB and glucose depletion do not cause the changes in cell behavior associated with cytochalasin treatment. Pretreatment of HeLa cells with 3′-deoxyadenosine also markedly inhibits their contractile and zeiotic response to cytochalasin (S. G. Sawicki, personal communication).

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