

# ACTION OF CYTOCHALASIN D ON CELLS OF ESTABLISHED LINES

## III. Zeiosis and Movements at the Cell Surface

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### ABSTRACT

The projection of knobby protuberances at the cell surface (zeiosis) is a general cellular response to cytochalasin D (CD), resulting from herniation of endoplasm through undefended places of the cortex during cell contractions and displacement of microfilaments induced by CD. Zeiosis is prevented by agents that interfere with the contractile response to CD, such as inhibitors of energy metabolism or cyclic AMP. The developed protrusions, which remain relatively stable in the presence of CD, contain chiefly mono- or subribosomes, and occasionally other organelles normally resident in endoplasm; compact microfilament felt occupies their bases and extends into their proximal stalks. Protein synthesis in the knobs is less than half of that in the polyribosome-containing endoplasm residual in the main body of the cell.

Knobs first protrude singly near the margin of the contracting cells and rapidly cluster into small groups in the periphery even at lower temperature. The clusters then migrate centripetally and coalesce into a single large aggregate near the apex of the immobilized and retracted cell: this movement is energy- and temperature-dependent. Aggregation is more prominent and stable in cell lines of epithelial derivation than in fibroblastic or other lines in which nuclear extrusion occurs more readily. The latter is regarded as a special manifestation of zeiosis.

Macromarkers, such as latex spherules, migrate like the zeiotic knobs on the cell surfaces in the presence of CD. The aggregated knobs, although persistent for days in the presence of CD, are rapidly recessed after withdrawal of the agent as ruffling is resumed and the cells spread. These movements are discussed in terms of current concepts of mobility of the cell membrane.

Most arresting among the visible effects of the cytochalasins on cells in monolayer are the cessation of locomotory and membrane movements (12, 65), the expulsion of their nuclei (12), and the

eruption of bulbous protrusions<sup>1</sup> at the surface (48, 38, 25). The latter are manifestations of a zeiotic process, more evident in some cell types than in others. We have attempted to relate these and some of the diverse phenomena resulting from treatment with CD to a single action of this agent on the contractile apparatus of the ectoplasm, producing a state of contracture accompanied by a redistribution of thin microfilaments (38, 39). In this communication we describe the zeiosis caused by CD, the cell types and circumstances in which it occurs, and the agents that affect it. Especially remarkable are the directed movements occurring at the surfaces of cells exposed to CD, for which the zeiotic knobs serve as a kind of macromarker. Their displacement is compared with the flux of other markers at the cell membrane, and the influence of temperature and of various drugs on the movements of the membrane are considered. These experiments provide some evidence on the ways in which the organization of the cell cortex in relation to surface-bound structures is modified by CD and other agents, and may offer some insights into mechanisms that govern movement at the cell membrane.

## MATERIALS AND METHODS

### Cells

The following cell types were studied: lines of epithelial derivation were HeLa (CCL2, American Type Cell Culture Collection, Rockville, Md.), HeLa S3 (Grand Island Biological Co., Grand Island, N. Y.), HEP2 (CCL23), and KB; lines of fibroblastic origin were PR105, L (mouse); (NCTC clone 929), and XC (established from a Rous sarcoma virus-induced rat tumor); also the MDBK (CCL22) and Vero (CCL81) lines originating from kidney cultures. All cells were grown in monolayer as hitherto described (38) except for HeLa S3, which was grown in suspension.

### Microscopy

Preparative methods for scanning or transmission electron microscopy have been given in Miranda et al.

<sup>1</sup> Such protuberances at the cell surface are often referred to in the recent literature (e.g., 28, 45, 54, 62, *inter alia*) as "blebs," and the process of their protrusion as "blebbing." In pathology, bleb (as synonymous with bulla) denotes a blister or bubble containing watery fluid. Hydropic or cystic vacuoles analogous to bullae can indeed bulge at the cell surface in some cytopathic conditions. To distinguish these from the protrusions described in this and the other cited reports, the authors have preferred descriptive designations such as knobs and protuberance, to the term "bleb."

(38, 39). For examination of living cultures, cells were planted in modified Cooper dishes (38). Subconfluent HeLa cell monolayers exposed to 0.25  $\mu\text{g}/\text{ml}$  of CD or dimethyl sulfoxide (DMSO) for 1 h were fixed in a methanol-Formalin-acetic acid mixture (85:10:5, vol/vol) and stained for DNA with the Feulgen reaction, for RNA with azure B, and for protein with acid fast green according to the methods prescribed by Deitch (16).

### Particles for Surface Movement

Latex beads of 0.8  $\mu\text{m}$  (Coulter Electronics, Inc., Fine Particle Group, Hialeah, Fla.) suspended in a balanced saline solution were applied to the medium of subconfluent monolayers of HeLa, HEP2, PR105, or XC cells growing in Cooper dishes modified for observation in phase contrast (38). After 15 or 30 min at 37°C, the cultures were rinsed vigorously three times with growth medium at 37°C. The effects of CD or Colcemid (Grand Island Biological Co.) on the migration of the beads were examined as indicated in the legends to Figs. 10-11.

### Active Agents

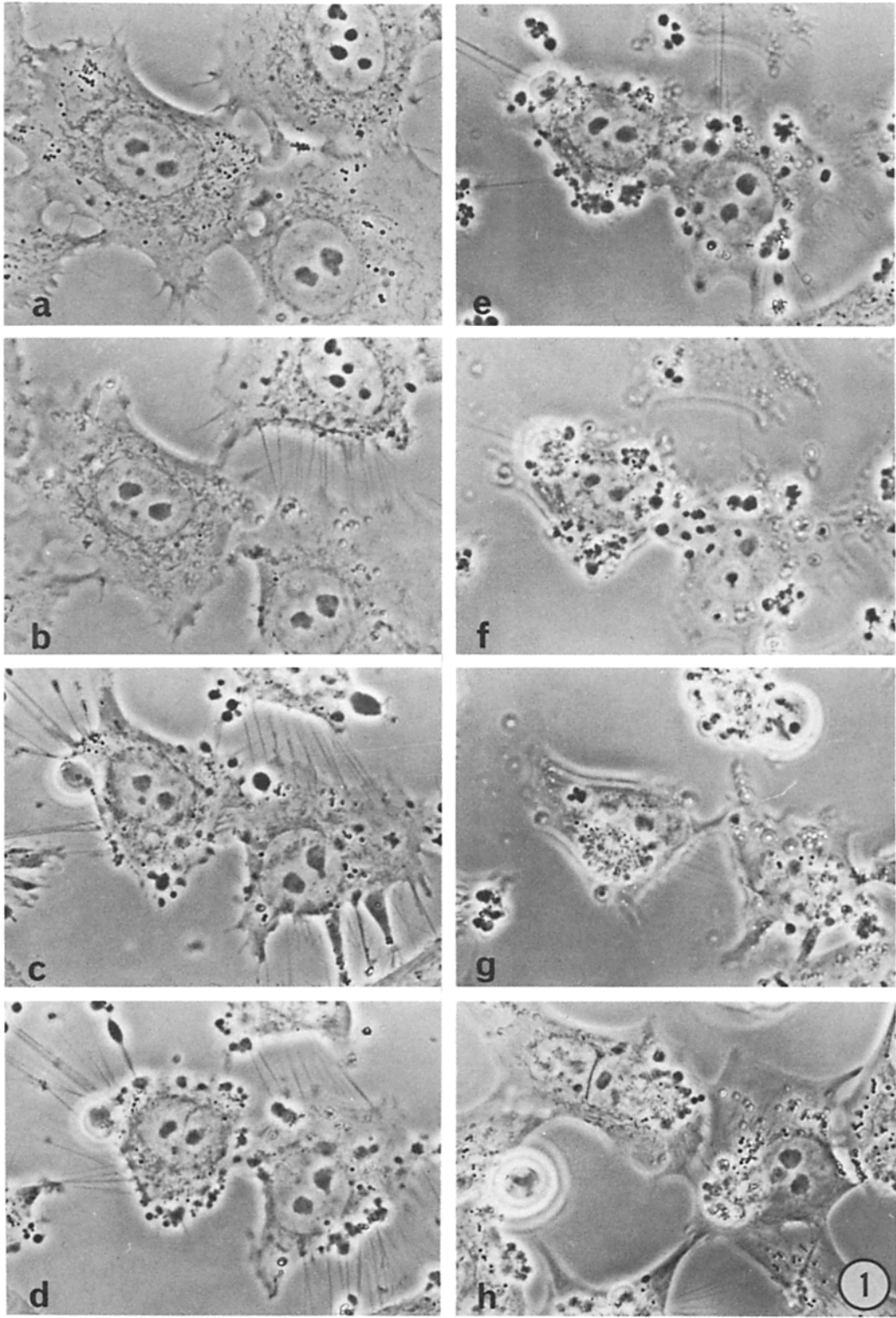
**CYTOCHALASIN D:** Details of preparation and treatment with cytochalasin D and/or DMSO have been described (38).

**COLCEMID:** Stock solutions of Colcemid of 10  $\mu\text{g}/\text{ml}$  were prepared in phosphate-buffered saline (Grand Island Biological Co.) and diluted in culture medium to a final concentration of 0.1 or 0.6  $\mu\text{g}/\text{ml}$ .

**CYCLIC AMP AND GMP:** N<sup>6</sup>O<sup>2</sup>-dibutyryl adenosine 3',5'-cyclic monophosphoric acid (dbcAMP) or O<sup>2</sup>-monobutyryl guanosine 3',5'-cyclic monophosphoric acid (mbcGMP) (Sigma Chemical Co., St. Louis, Mo.) and 1,3-dimethyl xanthine (theophylline) (Sigma Chemical Co.) were dissolved in growth medium at the concentrations indicated in Table III.

**UNCOUPLERS AND INHIBITORS OF ENERGY METABOLISM:** To evaluate the effects of these agents on zeiosis, monolayers were pretreated with inhibitor in a glucose-free medium for 6 min; CD was then added to a concentration of 0.2  $\mu\text{g}/\text{ml}$  and incubation continued for 15 min at 37°C. Controls included cells treated for 15 min with CD, 0.025% DMSO, or inhibitor alone in a glucose-free medium. The percentage of 1,000 cells exhibiting zeiosis was counted.

To evaluate the effects of inhibitors on the migration and clustering of preformed zeiotic knobs, monolayers were treated with 0.2  $\mu\text{g}/\text{ml}$  of CD for 60 min (*A*), or 100 min (*B*), in a glucose-free medium. Inhibitor was then administered to some of the cultures that had been exposed to CD for 60 min, in the continued presence of CD, for 40 min. Controls included cultures treated with DMSO or inhibitor alone. The proportion of 1,000 cells exhibiting a large apical aggregate of knobs was counted in each sample. Inhibition was calculated by comparing inhibitor-treated samples (*I*), with controls treated with CD alone for 60 min (*A*), and for 100 min (*B*), according



to the following formula, which corrects for the number of cells with aggregates before addition of inhibitor: % inhibition =  $100 - [(I-A/B-A) \times 100]$ .

The inhibitors were: 2-deoxyglucose (DOG), 2,4-dinitrophenol (DNP), antimycin A (AMA), carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), iodacetamide (IAA), sodium azide (NaN<sub>3</sub>) (Sigma Chemical Co.), and potassium cyanide (KCN) (Fisher Scientific Co., Fair Lawn, N.J.).

Monolayers treated with the agents as listed in Table II were rinsed with Earle's balanced salt solution (EBSS) at 37°C, fixed with 2.5% phosphate-buffered glutaraldehyde (pH 7.3), mounted on slides in phosphate buffer and scored with phase optics.

### Autoradiography

Subconfluent cultures were treated with 0.25 µg/ml CD for 90 min. The cells were then exposed to 1.0 and 10 µCi/ml of [<sup>3</sup>H]L-leucine (specific activity 5 Ci/mmol) for 5 or 10 min, in the presence of CD. The cultures were rinsed with EBSS, and fixed with 2.5% phosphate-buffered glutaraldehyde, osmicated, pelleted in agar, and embedded in Durcupan (Fluka AG, Buchs, Switzerland). Half-µm sections cut with a Porter-Blum MT2 ultramicrotome (Ivan Sorvall, Inc., Newtown, Conn.) were mounted on slides and prepared for autoradiography by dipping in a K5 emulsion (Ilford Ltd., Ilford, Essex, England). Autoradiographs were photographed with phase optics, magnified × 1200 and the number of silver grains in 25 cells per unit area was determined with the aid of a 0.5-cm<sup>2</sup> superimposed grid. The concentration of grains over the residual cytoplasm of the main cell body was compared with that overlying zeiotic aggregates.

### Incorporation of [<sup>14</sup>C]Amino Acids

HeLa cultures were treated with 0.35 µg/ml of CD or 0.03% DMSO for 2 h and then exposed to 1 µCi/ml of

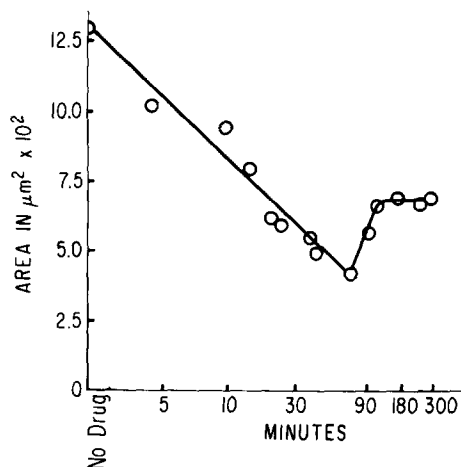


FIGURE 2 Sequential planimetric measurements of the area occupied by a HEP2 cell treated with 0.35 µg/ml CD as depicted in Fig. 1.

[<sup>14</sup>C]amino acid mixture (specific activity 57 mCi/mg; Amersham/Searle Corp., Arlington Heights, Ill.) in the presence of CD or DMSO, for 30 min. The cells were rinsed three times with fresh growth medium, once with EBSS. The cells were then trypsinized, precipitated with 10% TCA, dissolved in 1 N NaOH, neutralized with 1 N HCl and redispersed in 10% TCA, collected and washed on B-6 membrane filters (Schleicher & Schuell, Inc., Keene, N. H.), dried and counted in a Packard Tricarb liquid scintillation Spectrometer (Packard Instrument Co., Inc., Downer's Grove, Ill.).

## RESULTS

### Overview

With the onset of contraction (Figs. 1, 2), in 2–3 min after application of CD (range: 0.05–0.5

FIGURE 1 Sequence of eight frames showing the progression of visible events in the same HEP2 cells during 18 h of exposure to 0.35 µg/ml of CD. Phase contrast, × 740. (a) Three normal HEP2 cells in monolayer. (b) The same field 3 min after addition of CD. Retraction has begun but cells remain in contact at points of adherence. Densities at the margin are foci at which zeiotic protuberances are forming. (c) The same field at 10 min. Taut processes (retraction fibres) anchor the retracting cell to the surface. Dense protrusions burgeon at the cell margins. (d) At 30 min there is marked mutual retraction and numerous zeiotic knobs protrude at the free margins. (e) At 90 min the zeiotic processes are beginning to cluster. (f) At 105 min the zeiotic protrusions are in discrete clusters. The nuclei now protrude above the focal plane. (g) The same field at 5 h. The clusters of knobs have migrated to the top of the free surface and are massed in a large single aggregate or bouquet, in the region of the protruding nucleus. (h) The same field at 18 h. The cells have spread out to occupy some of their original area. They have characteristic polygonal shapes and straightened margins. In the interval since frame (g) the cell at left has completed karyo- but not cytokinesis and is now binucleate. The nuclei of the central cells have resumed their usual positions, but the nucleus of the cell at lower left (surrounded by phase halos), however, its virtually extruded. The massed zeiotic knobs (bouquets) remain immobile on the top side.

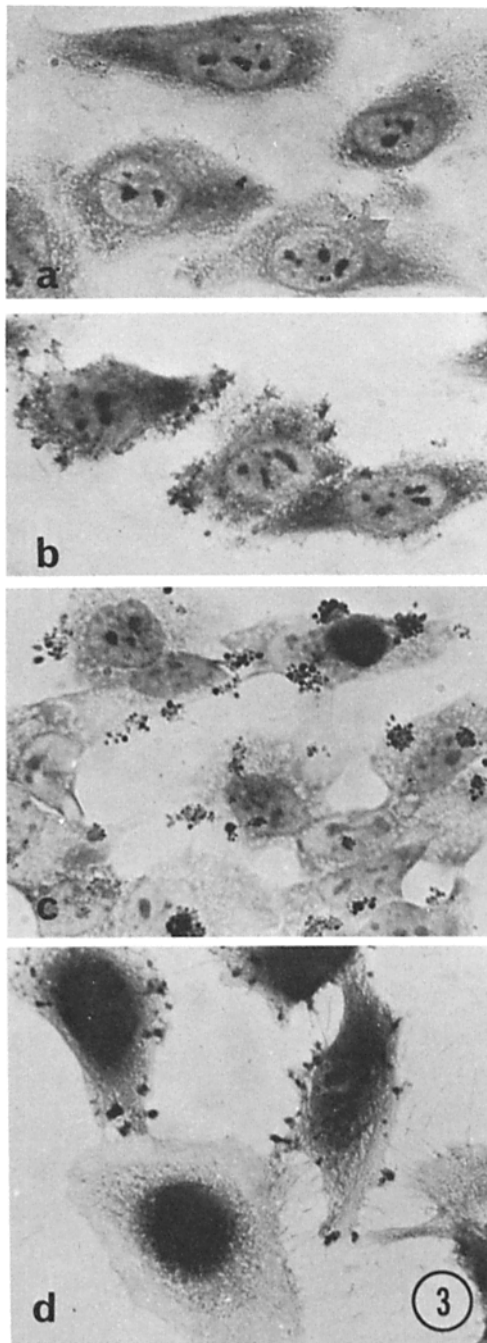


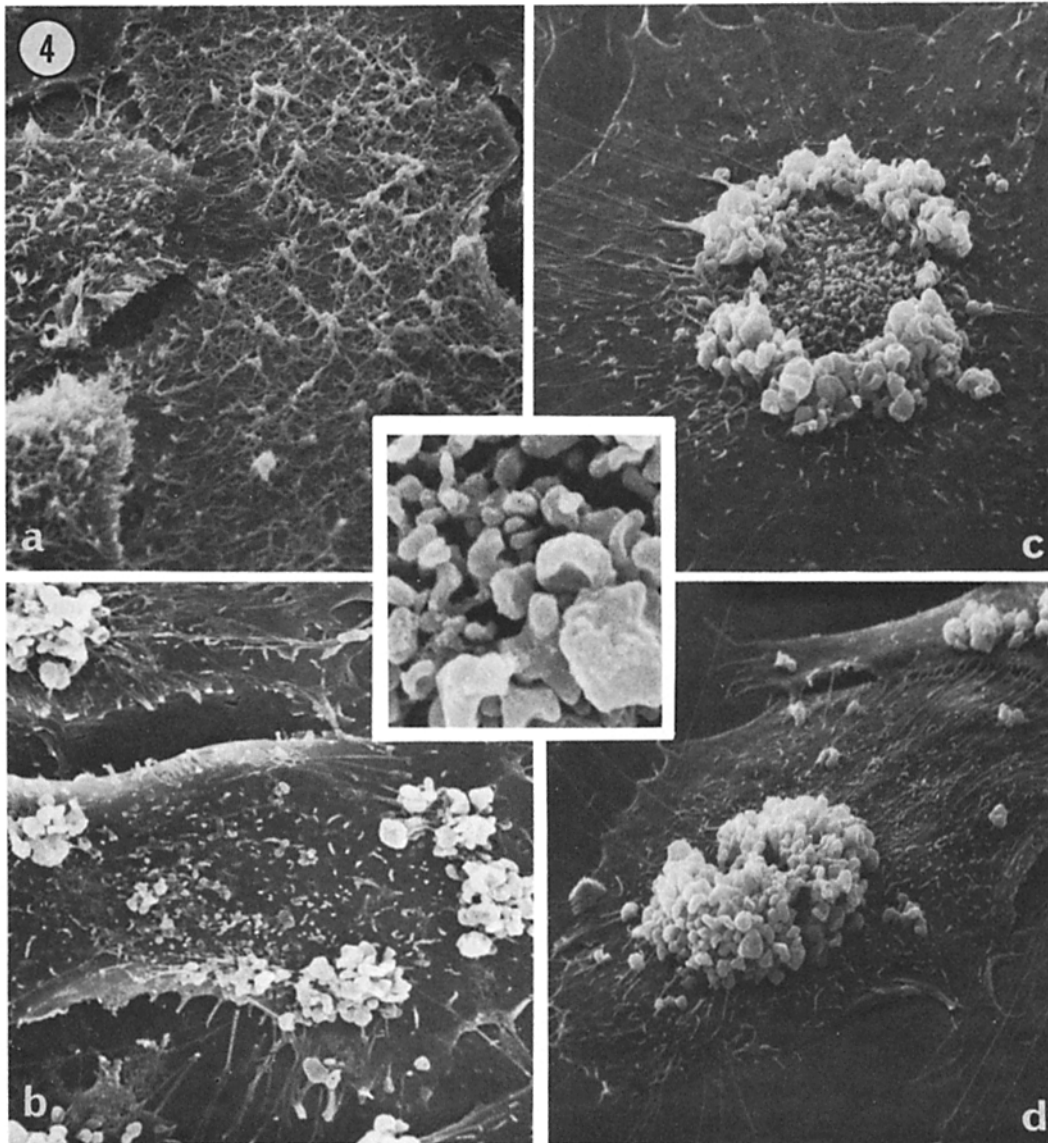
FIGURE 3 HeLa cells stained with azure B for RNA. (a) Control cells in 0.02% DMSO medium; (b) cells in 0.5  $\mu\text{g/ml}$  CD for 1 h, and (c) for 5 h. (d) HeLa cells in 0.25  $\mu\text{g/ml}$  CD for 15 min, stained with Feulgen fast green for DNA and protein. The intense staining of the zeiotic processes by both basic and acidic dyes is attributable to their high concentration of ribonucleoprotein contained

$\mu\text{g/ml}$ ) microvilli are withdrawn all over the cell surface and blunt umbonate processes are protruded all about the periphery (Figs. 1 a-c, 3, 4). At first hemispherical, they subsequently enlarge and develop clavate or bulbous ends on erect, narrow stalks, thus assuming the form of knobs (Figs. 5, 6). These migrate on the cell surface, first coming together in small peripheral clusters which move centripetally and coalesce into larger groups (Figs. 1 e-f, 4 b). In HeLa, KB, and HEP2 cells, by 60-90 min of exposure to CD, the knobs have massed at or near the cell apex in one or sometimes two large aggregates ("bouquets" or "crowns") (Figs. 1 f-h, 4 c, d). Some nuclear protrusion at the apex is also evident by this time in HeLa, HEP2, and Vero cells, especially at the higher concentrations of CD. The events proceed to completion more rapidly and are most readily visualized in the HeLa, HEP2, and KB lines. Their incidence, extent, and dose dependence differ in other cell types, and these variations will be considered below.

#### *Genesis and Development of Zeiotic Processes*

In normal, untreated cells the microfilament web of the cortex is a more or less continuous barrier between the endoplasm and the inner face of the plasma membrane. Concomitant with the beginning of cell contraction, the thin subplasmalemmal microfilaments become compacted into larger focal accumulations in the cortex, a process that entails their displacement and detachment from some patches of the cell membrane. This apparent discontinuity in the cortical web may deprive some sectors of plasmalemma of an adequate filamentous net, and probably allows endoplasm to flow up to the unprotected inner aspect of the cell membrane. These plugs of endoplasm expressed during the contraction then burgeon out at the periphery of the topside as the early zeiotic knobs. In the presence of 0.4 M sorbitol, zeiosis cannot be elicited by exposure to efficient concentrations of CD (0.33  $\mu\text{g/ml}$ ), but within minutes after withdrawal of the sorbitol, marked zeiosis again occurs in response to CD. This result suggests that increased intracellular hydrostatic pressure promotes zeiosis, and that this is pre-

in ribosomes (see Figs. 6 and 8). Basophilia of the remaining cytoplasm diminishes during exposure to CD.  $\times 750$ .



**FIGURE 4** Scanning electron micrographs of HeLa cells in monolayer, before and during exposure to 0.25  $\mu\text{g}/\text{ml}$  of CD. (a) Normal cells in control medium containing 0.025% DMSO. The microvilli of the top surface are densest over the central part.  $\times 2,200$ . (b) After exposure to CD for 45 min the recessed microvilli of the contracted cells are represented by a stubble on the surface. The CD-induced zeiotic knobs are in several discrete clusters near the cell margins.  $\times 2,200$ . (c) After exposure to CD for 30 min the clusters of knobs have migrated centripetally and aggregated; they form a collar about the convexity overlying the nucleus. The surface of this enclosed area is beset by short, blunt, presumably microvillous rests. At the margins of the collar of knobs there are a number of short processes each of which splays out into several long filar retraction fibers which course over the top surface and attach to the substratum. A sparse stubble of recessed microvilli is evident on the surface of the attenuated peripheral part of the cell.  $\times 2,200$ . (d) After exposure to CD for 4 h, the clusters of knobs, having migrated centrally, are aggregated into a prominent bouquet or crown at the apex. Microvillous rests, and the long filar retraction fibers radiating from the contracted crown, are seen on the top surface of the extended peripheral cytoplasm.  $\times 2,200$ . *Inset*, Detail, at higher magnification of the top of the bouquet of knobs after 90 min of exposure to CD. The expanded ends of most of the knobs are flattened or scaphoid.  $\times 6,400$ .

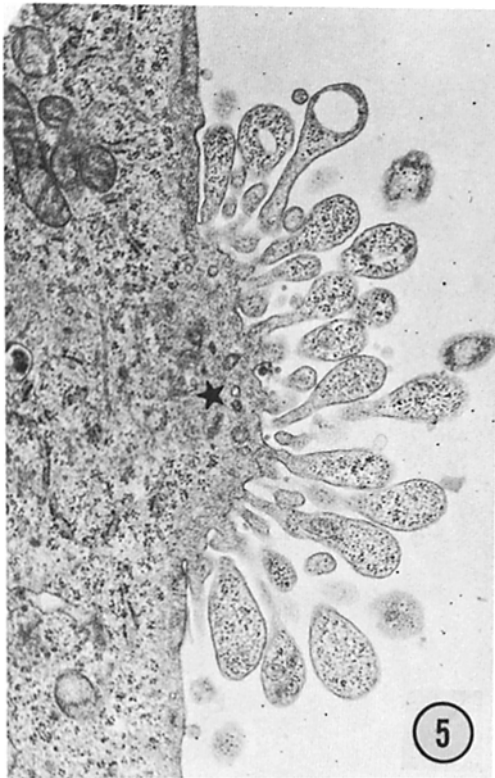


FIGURE 5 Section through a zeiotic cluster after exposure to  $0.25 \mu\text{g/ml}$  of CD. The erect knobs take origin from a convexity or hillock occupied by a compact microfilarmentous felt (star), some of which extends into the bases of the stalks.  $\times 8,800$ .

vented by osmotically active agents that decrease internal pressure.

Presumably, as intracytoplasmic pressure is increased during the development of contracture (Fig. 2) more endoplasm is herniated, and the protrusions become typically knoblike. As this occurs, the adjacent pads of compacted microfilaments move together in the direction of the outward flux, ultimately to occupy the cortex at the base of the knob, and to extend part of the way into its peduncle (Figs. 5–8). As the zeiotic knobs migrate and cluster in groups (Fig. 1 *f*, 4 *b*) they appear to be accompanied by their associated mats of filament felt; in any case the felt is coalesced in large pads subjacent to the clusters, and the knobs (Figs. 6, 8) emerge from a convexity or hillock occupied by a massed felt of filaments (Fig. 5).

The protrusion of zeiotic knobs, like general cell contraction, is temperature dependent. Below about  $20^\circ\text{C}$ , zeiosis induced by CD is markedly inhibited. In response to  $0.33 \mu\text{g/ml}$ , a concentration that elicits zeiosis in some 95% of HeLa cells at  $37^\circ\text{C}$  in 15 min, only 50% of cells exhibit knobs at  $12^\circ\text{C}$  even after 180 min and at  $6^\circ\text{C}$  only 4% of cells are knobbed after 180 min.

#### The Zeiotic Knob

The fully formed knob is erected on a slender stalk, sometimes branched, and terminates in a dimpled or uniconcave expansion (Fig. 4 inset). The peduncle is filled to about three-fourths of its extent with microfilament felt: parallel filaments

FIGURE 6 Transverse section of a HeLa cell bearing a zeiotic aggregate after 5 h of exposure to  $0.25 \mu\text{g/ml}$  of CD. The cortex from which the cluster of erect knobs arises is mostly occupied by contiguous masses of compact microfilarmentous felt forming a pad more than  $1 \mu\text{m}$  in thickness. Masses of this felt extend deep into the endoplasmic area, and into the bases of the stalks of the zeiotic knobs. In the cortex subjacent to plasma membrane, smooth-walled vesicles (*v*) are rare but bristle-coated vesicles (*cv*) are numerous. The zeiotic protuberances (*z*) contain endoplasm, which here, in absence of a cortical web, abuts on the inner face of plasma membrane. Most of the endoplasmic content of the distal expansions of the knobs consists of monoribosomes (and some subunits), and occasionally, elements of rough endoplasmic reticulum (*rer*). The endoplasm residual in the cell body below and between the masses of microfilament fleece appears to contain only polyribosomes (ribosome clusters); multivesicular bodies (*mv*) are also prominent within it. The filament felt in the cortex of the flat basal aspect of this cell is apparent at bottom.  $\times 28,000$ .

FIGURE 7 Diagram showing (a) the normal microfilament net of the cortex, and (b) its contraction and condensation some minutes after application of CD, and its retraction from some loci, more or less denuding the inner face of the plasmalemma of cortex; here, endoplasm abuts on plasma membrane and burgeons out (zeiosis) (arrows). (c) As knobs are formed by further protrusion of endoplasm and begin to cluster, the subjacent felt of compacted filaments coalesces into larger masses and extends up into the stalks.

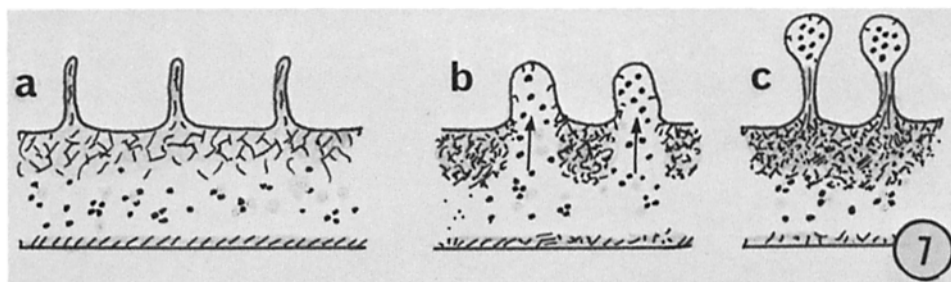
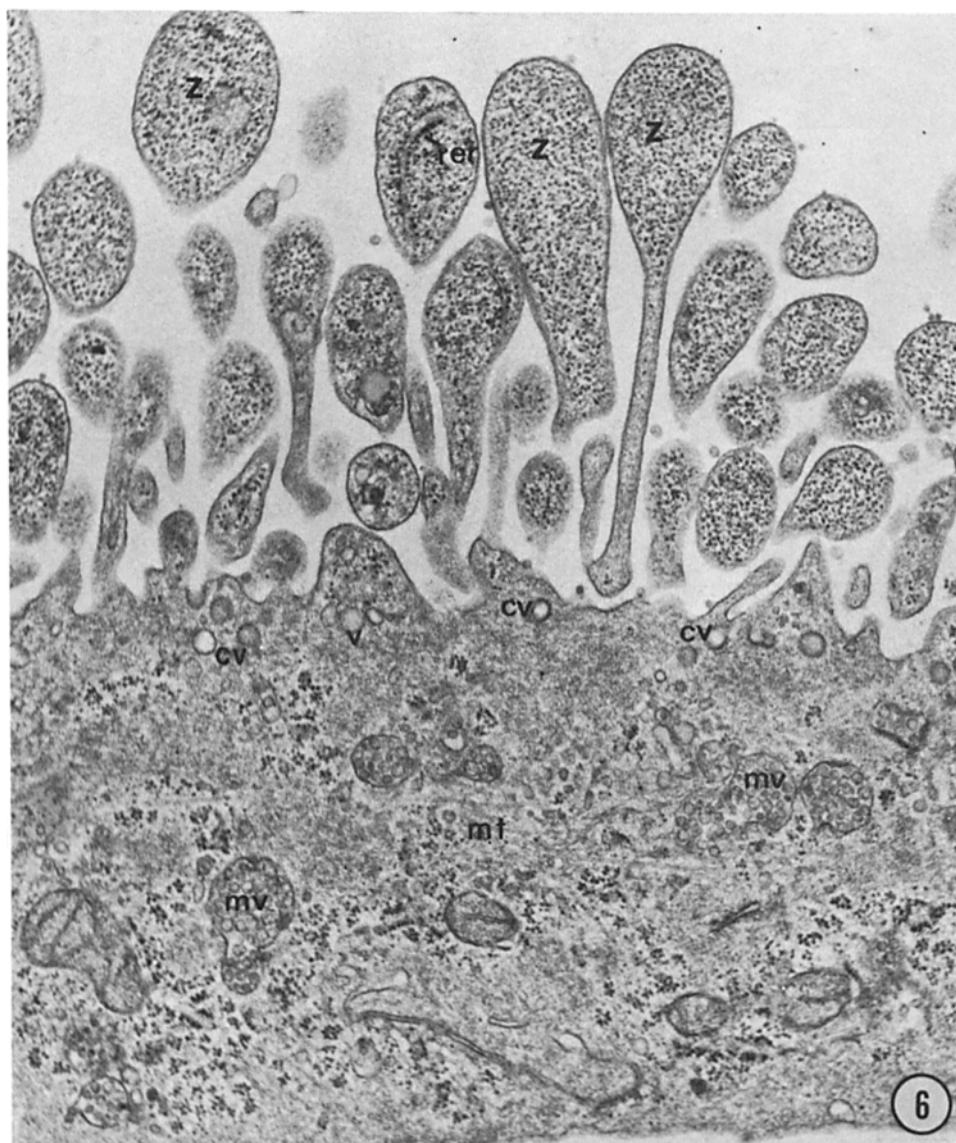






FIGURE 8 Section through a HeLa cell in  $0.25 \mu\text{g/ml}$  of CD for 6 h. A large collection or bouquet of zeiotic knobs (like that depicted in Figs. 1 g or 4 d) is aggregated on top and toward the periphery of the cell. In addition to the usual component of monoribosomes, the endoplasm in the protuberances contains pieces of rough and smooth endoplasmic reticulum (*rer*), some vesicles, and mitochondria (*m*). Membrane-bounded vesicular bodies (*v*) ranging from 20 to 80 nm, some with dense contents, lie free among the knobs. The cluster of knobs arises from a convexity formed by a thick, compact microfilament felt (stars), continuous with that underlying the plasma membrane of adjacent areas of both top and base of this cell. Dense bodies (*db*) considered to be Z bodies, are present in or near this felt, within which they develop. A big multivesicular body (*mv*), microtubules (*mt*), polyribosome clusters, mitochondria (*m*), and nucleus (*N*) are readily identifiable in the endoplasm.  $\times 22,500$ . Inset: A zeiotic protrusion at higher magnification, showing the microfilament fleece in its base and peduncle and the paucity of subplasmalemmal microfilaments in the clavate end. It contains endoplasm with many mono- and subribosomes. A coated vesicle is marked *cv*.  $\times 66,000$ .

or microtubules are but rarely discerned (Figs. 5–8). The terminal bulb contains endoplasm represented by dispersed monoribosomes and some bodies of <10 nm, interpretable as ribosomal subunits. Segments of rough or smooth endoplasmic reticulum and some small vesicles are frequent in these contents; occasionally mitochondria, lysosomal elements, glycogen granules, and lipid globules are also present. Polyribosomes are seldom discerned in the knobs, but they are numerous in the endoplasm residual in the cell body in which indeed, there are but few dispersed monoribosomes. This endoplasmic content of the knob is intermittently separated from the dense line representing inner leaflet of plasma membrane by a lamina of low density about 10 nm wide. In some other places, endoplasm appears to abut directly on plasma membrane.

The zeiotic knobs are extremely phase dense from the time of their first formation (Fig. 1) and stain intensely with cationic dyes in conditions selective for RNA, and with acid dyes in conditions selective for protein (Fig. 3). As protrusion and clustering of knobs proceed in the presence of CD, the apparent basophilia of the residual cytoplasm diminishes; the intensely colored clusters of knobs stand out in striking contrast (Fig. 3 c). The density and intense stainability of the knobs reflects the very high concentration of ribonucleoprotein, evidently ribosomal, in them. This persists for as long as the knobs remain intact, up to 72 h in CD on HeLa, HEP2, or Vero cells. Notwithstanding the redistribution of ribosomes, the overall incorporation of [<sup>14</sup>C]leucine into protein in HeLa is only diminished by 15–20% after 1 h in CD. The site of incorporation of [<sup>3</sup>H]leucine revealed in autoradiograms is predominantly in the residual endoplasm of the cell body (which harbors the polyribosomes); there is less than half the apparent protein synthesis (i.e., number of grains per square micrometer) in the zeiotic protuberances.

#### *Movement and Aggregation of Knobs on the Cell Surface*

Zeiotic knobs are first protruded at the periphery of the contracting cell, at or near the margins, and often from the straightened cell retraction fibres (Figs. 1, 4). Initially, they would appear to ride more or less randomly in the peripheral domain, and to cohere when they impinge. In this way, first smaller, and subsequently larger tight clusters of knobs are formed on the surface about

the entire periphery (Figs. 1 a–f, 4). This is the picture usual in HeLa, HEP2, and KB after 30–40 min in moderate concentrations (ca. 0.5 µg/ml) of CD. Thereafter, the overall vector of movement of the clusters on these cells is more rapidly centripetal with some lateral play, however, as the marginal zone is retracted. The process of coalescence of the clusters proceeds as they make contact, and by 90–180 min, a big aggregate of all the clusters (the bouquet or crown) will have formed at or near the cell apex (Figs. 1 h, 4 c, d). It remains fixed in this location as long as the cell is exposed to CD. The average rate of centripetal movement of clusters along the radial vector, resulting from translation on the surface and overall contraction, is estimated to be about 3.5–4 µm/min at 37°C, with reference to the original cell margin. Once zeiotic knobs have been induced about the cell margins by brief treatment with CD at 37°C, their movement in the peripheral or subapical area, and the formation of small clusters of knobs, can evidently proceed at lower temperatures albeit at a somewhat slower rate, even at 6°C. But their centripetal migration and the aggregation of clusters into a big crown or bouquet at the apex, is slowed below 37°C and almost prevented below 12°C (Table I).

Zeiosis in cells in suspension (HeLa S3) exposed to CD is also accompanied by withdrawal of their luxuriant microvilli and apparent cell contraction, and is followed by migration and formation of a large cluster of knobs at one sector of the round cell (Fig. 9).

#### *Macromarkers*

Both the direction and rate of migration of the zeiotic knobs are similar to that of marker macroparticles in presence of CD. Latex spheres of 1.8 µm, adhering to the surfaces of normal HeLa cells, move irregularly; although the overall vector of movement tends toward the apex, the beads do not progress along a radial line, nor do they accumulate in a tight central cluster. In the presence of CD (0.25 µg/ml) the direction of movement of particles previously applied to the cells is generally centripetal and the particulate markers have mostly gathered in clusters at the top of the cell by 60–90 min (Fig. 10 a, b). The clusters remain relatively immobile in this location after withdrawal of CD has allowed respreading, flattening, and resumption of ruffling and locomotory movement (Fig. 10 c). Few or no latex spherules adhere

TABLE I  
Effect of Temperature on Movement and  
Aggregation of Zeiotic Knobs Induced by CD  
in HeLa

Temp.	Time at low temp.	Total time in CD	Cells with dispersed knobs	Cells with clusters	Cells with "crowns"
°C	min	min	%	%	%
37	—	15	69	19	4
	—	45	34	35	23
	—	90	6	26	64
	—	180	8	22	66
22	30	45	43	46	6
	75	90	17	53	24
	165	180	15	45	36
12	30	45	29	53	9
	75	90	36	45	13
	165	180	31	46	18
6	30	45	42	39	9
	75	90	45	42	7
	165	180	35	47	10

HeLa cell monolayers were incubated in 0.25  $\mu\text{g}/\text{ml}$  CD for 15 min at 37°C to elicit zeiotic knobs. At this point, the knobs are randomly and simply dispersed chiefly about the cell periphery near the margins. Cultures were then maintained at the lower temperatures indicated in the table, and 1,000 cells scored for the occurrence of zeiosis, indicating (a) the percent of cells with dispersed knobs, (b) the frequency of cells with multiple small clusters (like those in Figs. 1 e, f, or 4 b), (c) the numbers of cells with big, usually single, apical aggregates or "crowns" (like those in Figs. 4 c, d, 10, 1 g), at the times shown in the table.

to the surface of cells (whether of epithelial or fibroblast derivation) that have first been treated with CD (0.5  $\mu\text{g}/\text{ml}$  for 3 h or more), especially after maximal zeiosis and clusters of zeiotic knobs have been allowed to develop.

Applied to Colcemid-pretreated HeLa (0.6  $\mu\text{g}/\text{ml}$  per 7 h), in the continued presence of 0.6  $\mu\text{g}/\text{ml}$  Colcemid, the latex spherules migrate centrally to the apex at about 3–4  $\mu\text{m}/\text{min}$  (Fig. 11 a, b). The addition of 0.25  $\mu\text{g}/\text{ml}$  of CD to the Colcemid-treated cells does not greatly alter either the direction or rate of particle flow on the cell surface, and single central clusters already formed in presence of Colcemid alone remain evident after adding CD (Fig. 11 c). Also, the presence of Colcemid does not affect the distribution of beads

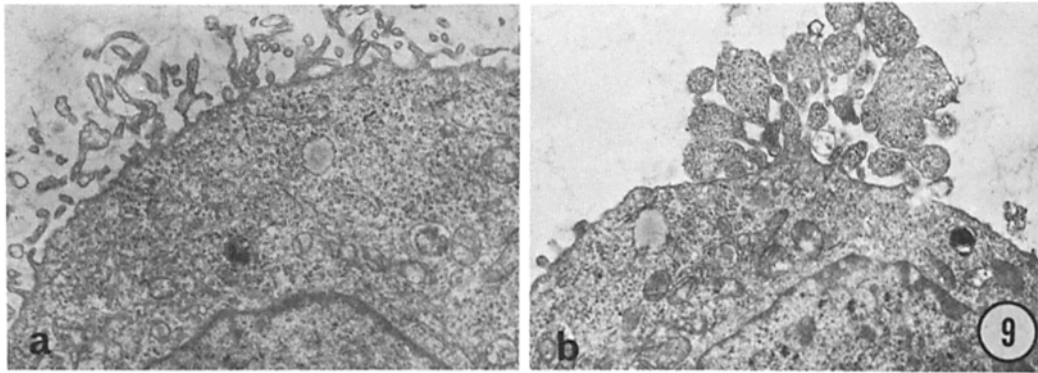
either after spreading and recovery from CD (Fig. 11 d) or in cells which had not been treated with CD.

#### Fate of Zeiotic Protrusions; Recovery from CD

The large apical bouquet (or zeiotic crown) of HeLa rests more or less immobile and most of its constituent knobs relatively unchanged on viable cells for periods of 72 h or longer in moderate concentrations of CD (0.2–0.5  $\mu\text{g}/\text{ml}$ ). However, a few knobs, especially at their clavate ends, may begin to show regressive changes such as hydropic swelling or loss of internal structure at 5–6 h. In HeLa, small bodies (microvesicles or microspherules) of cytoplasmic origin and of heterogeneous size, (from 20 to 80 nm) may also become numerous at this time (Fig. 8). Some of these in the range of 20–30 nm contain a central dense granule which gives them an appearance of viruslike particles. They lie free at the cell surface or among the fronds of the clusters. These, and larger pieces of cytoplasm (up to 1  $\mu\text{m}$ ) staining intensely for RNA, dislodged by gentle agitation of the medium and evidently derived from zeiotic processes, indicate that some cytoplasmic constituents are lost by sloughing. Such loss is roughly dose dependent. But the main mass of knobs remain intact, and on withdrawal of CD undergo rapid retrocession and their contents are internalized *in situ*. This process of recovery is accompanied by redistribution of the underlying microfilament pad without translatory movement of the whole cell. By 90–120 min, this reversion is mostly completed, even after prolonged sojourn in CD, with the exception of some vermiform excrescent residua of unresorbed zeiotic knobs, on the surfaces of some cells. Their number is related to the concentration of CD, and especially to the duration of exposure.

#### Nuclear Protrusion

All cell lines observed exhibit some nuclear protrusion during exposure to moderate doses of CD. Protrusion of the nucleus is evidently a more extreme event than the exhibition of small zeiotic knobs in the herniation of endoplasmic contents through the cortex under the influence of CD. In most cells, the nucleus is at first displaced peripherally. Like some other organelles resident in the endoplasm (e.g., mitochondria), the nucleus may then be expressed and subsequently wholly deliv-



**FIGURE 9** (a) Section of a HeLa-S3 cell from a suspension culture grown in control medium containing 0.025% DMSO. Many long slender microvilli extend from the surface. The microfilaments of the cortex are disposed in a thin continuous layer (net) separating endoplasm from plasmalemma.  $\times 8,000$ . (b) Section of a suspended HeLa S3 cell after 3 h in CD. A burst of clustered zeiotic knobs arises from an eminence packed with filament felt. The remaining cell surface is smooth with only a few short blunt rests of microvilli. Few microfilaments now underlie the plasma membrane.

ered at the topside (Fig. 12), presumably through a gap in the cortical tunic of filaments, as intracellular turgor increases. The dose required for nuclear protrusion is higher than that which elicits ordinary knob formation in the various cell lines, except for L cells, in which nuclear protrusion and zeiosis begin more or less coincidentally. In general, nuclear protrusion reaches highest incidence only after doses that elicit maximal cytoplasmic contraction. Like other residents of endoplasm which become herniated, the nucleus is surrounded by an envelope of monoribosome-rich cytoplasm contained in a distended protuberance the contents of which are not separated from the enclosing plasmalemma by a barrier of microfilaments (Fig. 13), although feltlike subplasmalemmal accumulations of compacted microfilaments may be found at or near the neck of the protuberance. Like knobs, nuclei may remain protruded for long periods (48 h) whilst in moderate concentrations of CD, without much morphological change (except for some loss of interchromatinic density) and with only little loss of viability of the cells. Nuclei completely protruded at the surface of PR105, XC, or L cells are especially vulnerable to "spontaneous" detachment or extrusion, a separation promoted by agitation of the medium. Most cells of the other lines require application of gravitational force to separate them efficiently from their nuclei; 90–100% of Vero can thus be enucleated after moderate doses of CD, but not more than 40–50% of HeLa cells (S. G. Sawicki, personal communication).

Even after prolonged protrusion, nuclei, like most other zeiotic processes, are readily reinserted into the main body of the cell after withdrawal of CD. This retrocession cannot be completed in cells treated with Colcemid (q.v.).

#### *Effect of Various Agents on CD-Induced Zeiotic Phenomena*

Zeiosis, including nuclear protrusion, is probably a consequence, at least in part, of contractile tension developed in cells under the influence of cytochalasin, although CD-induced contracture does not necessarily cause zeiosis in all cell types. Some inhibitors of energy metabolism and cyclic nucleotides can inhibit contracture; the effects of these agents and of Colcemid on the zeiotic process have been investigated. In these observations, the (a) protrusion of endoplasm about the periphery at the cell surface (zeiosis), (b) clustering of knobs, and (c) migration of clusters and their coalescence into a big apical bouquet, have been regarded as separable events.

**INHIBITORS OF ENERGY METABOLISM:** Table II shows that concentrations of some of the inhibitors effective in depressing energy metabolism limit or suppress zeiosis induced by CD. Deoxyglucose, dinitrophenol, antimycin A, and the carbonyl cyanide compound CCCP, virtually prevent knobbing, but KCN,  $\text{NaN}_3$ , or iodacetate were only moderately effective as inhibitors of zeiosis. Iodacetate could only be applied within the specified time limits because of its cytotoxicity.

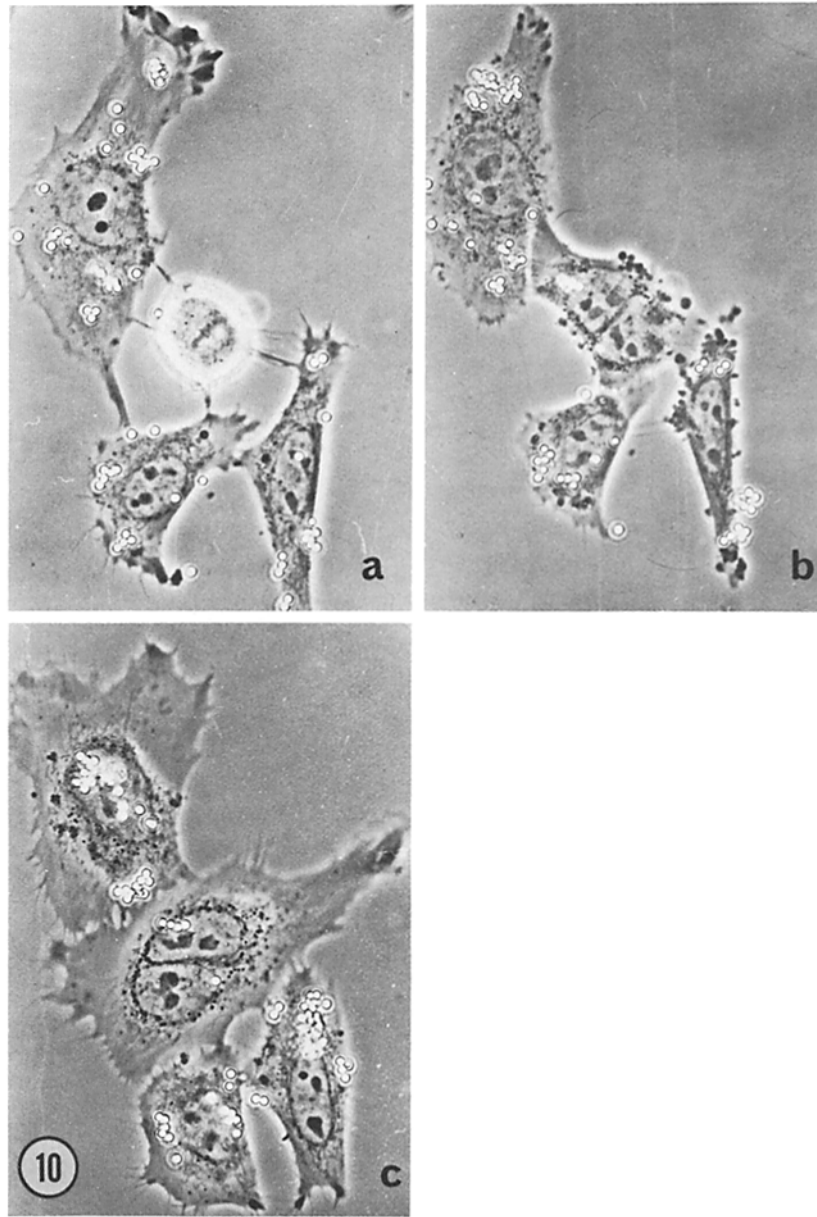


FIGURE 10 Migration of latex beads on surfaces of HEP2 cells in presence of CD. (a) 2 min after adding  $0.25 \mu\text{g}/\text{ml}$  of CD, showing distribution of latex spheres previously applied to the monolayer. The cells have begun to contract and to protrude some zeiotic knobs. The central cell is metaphase. (b) After 60 min in CD most spheres are aggregated into larger groups; some of these tend to move centripetally. The cells are more contracted and exhibit many knobs. The central cell, having completed karyokinesis but not cytokinesis, is binucleate. (c) Following withdrawal of CD after exposure for 2 h and at 90 min after addition of fresh drug-free medium, the cells have recovered and spread out. The spheres, having aggregated in clusters at or near the apex, remain fixed in that position. Phase contrast  $\times 640$ .

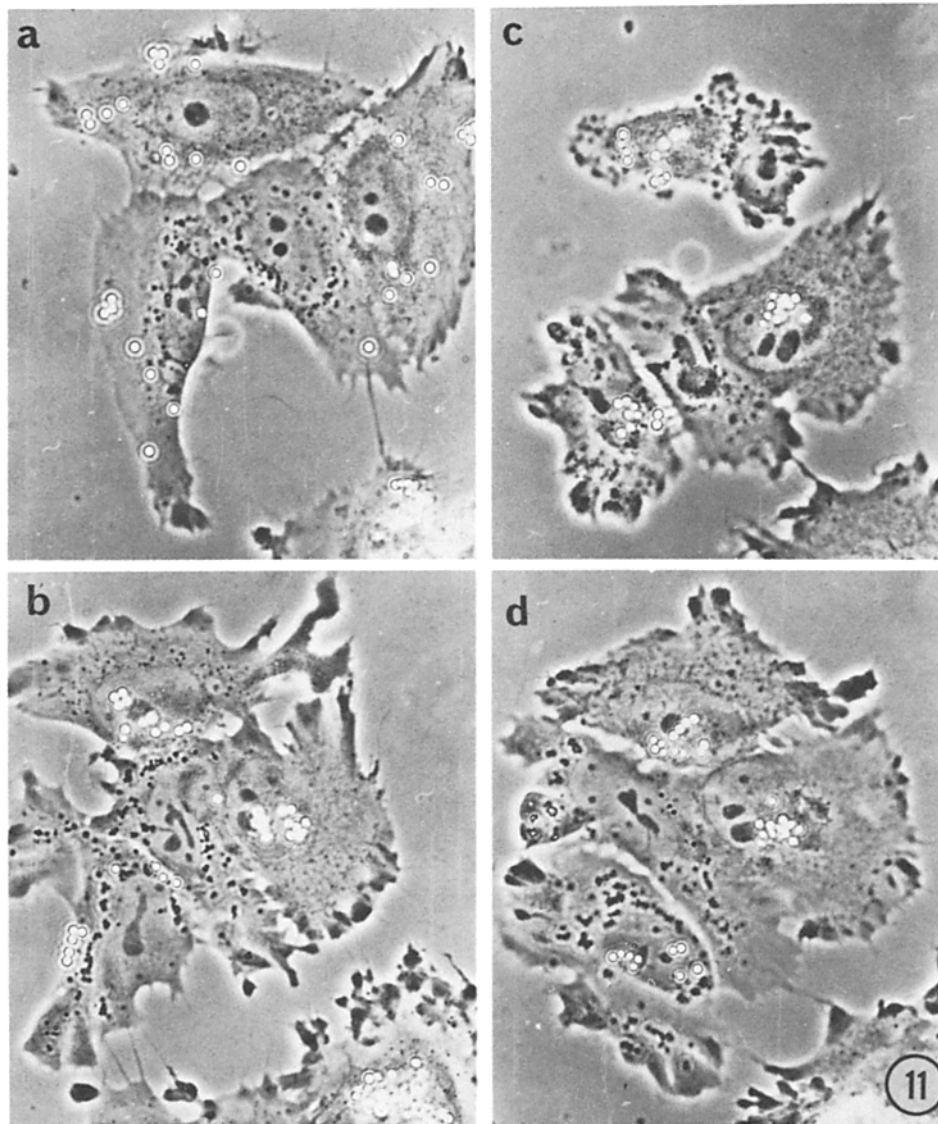


FIGURE 11 (a) A group of HEP2 cells which had been exposed for 7 h to  $0.6 \mu\text{g/ml}$  of Colcemid, 6 min after application of latex spheres (see Materials and Methods) in presence of Colcemid. The adhering beads are dispersed over the cell surfaces. (b) The same cells after 6 h of incubation in the Colcemid medium. The spheres have aggregated centrally at the apex. The peripheral cytoplasm is extenuated and many phase-dense ruffles are evident at the margins. (c) 20 min after addition of  $0.25 \mu\text{g/ml}$  of CD to the cells shown in Fig. 11 (b), in the continued presence of Colcemid; the cells have contracted, ruffles are withdrawn and numerous zeiotic knobs have erupted near their margins. The latex spheres remain in tight clusters at the apices. (d) The same group of cells, 60 min after withdrawal of CD, but in continued presence of Colcemid. The cells have respread and again exhibit exaggerated ruffling. The latex spheres remain aggregated at the apices. Phase contrast  $\times 700$ .

When zeiotic knobs are first expressed by pre-treatment with CD, and inhibitor is then applied in the continued presence of CD, the effect on the clustering and subsequent central aggregation of

clusters ("crowning") can be assessed by scoring the incidence of apical crowns. From Table II, it appears that those inhibitors most effective in preventing zeiosis are moderately effective in pre-

TABLE II  
Effect of Inhibitors of Energy Metabolism on CD-Induced Zeiosis and on Aggregation of Knobs in HeLa

Inhibitor	Concentration	Zeiotic cells	Inhibition of zeiosis	Cells with apical aggregates	Inhibition of aggregation
	<i>M</i>	%	%	%	%
None	—	70*	—	20† 81‡	—
DOG	10 <sup>-2</sup>	2	97	52	30
DNP	5 × 10 <sup>-3</sup>	2	97	63	39
AMA	10 <sup>-4</sup>	4	94	58	49
CCCP	2 × 10 <sup>-5</sup>	3	96	51	27
KCN	2 × 10 <sup>-3</sup>	39	45	—	—
IAA	10 <sup>-3</sup>	48	32	—	—
NaN <sub>3</sub>	10 <sup>-2</sup>	54	23	—	—
IAA + NaN <sub>3</sub>	10 <sup>-3</sup> + 10 <sup>-2</sup>	29	58	75	< 10

Inhibitors were evaluated for ability to interfere with CD-induced zeiosis, or with the subsequent migration and apical aggregation (crowning) of zeiotic knobs after their prior elicitation with CD, as described in Materials and Methods. The incidence of zeiosis was determined at 15 min after application of 0.25  $\mu\text{g/ml}$  of CD, with or without inhibitor. Aggregation (crowning) was scored in cultures in which knobs had been elicited (by prior treatment with 0.25  $\mu\text{g/ml}$  of CD for 60 min) after 40 min of exposure to inhibitor in continual presence of CD. The percent inhibition was calculated by comparing inhibitor-treated samples with controls treated with CD alone; the figures were corrected for the number of cells in which apical crowns had already formed at 60 min before the addition of inhibitor, according to the formula given in Materials and Methods.

\* At 15 min.

† At 60 min.

‡ At 100 min.

venting aggregation and migration of knobs already protruded. Under the conditions of the experiment, in which cells were not also pretreated with the inhibitors of energy metabolism (as they were in assaying the effect of inhibitor on zeiosis), it is probable that the maximal inhibitory effects of these agents were not exerted. Clustering of knobs at the periphery was less affected by the inhibitors than their centripetal movement and aggregation near the apex.

By 35 min after withdrawal of CD (e.g., 0.2  $\mu\text{g/ml}$  for 90 min), apical aggregates (crowns) of knobs which had been induced by CD will have dispersed in 75% of the cells, and by 60 min, 90% or more of the cells resume their normal shapes. The dispersion and recession of the zeiotic knobs leading to recovery is also markedly disturbed by inhibitors of energy metabolism. DOG is most

effective: a concentration of 10<sup>-2</sup> M applied to HeLa after withdrawal of 0.2  $\mu\text{g/ml}$  CD inhibits recovery by 80%; DNP, AMA, and CCCP are less potent and inhibit recovery by about 60%.

**CYCLIC NUCLEOTIDE:** Table III shows that treatment of HeLa with dibutyl 3',5'-adenosine monophosphate (cyclic AMP) together with the phosphodiesterase-inhibitor theophylline, markedly curtails their ability to respond to an effective concentration of CD by contracting, and profoundly inhibits their ability to exhibit zeiosis. In contrast, monobutyl 3',5'-guanosine monophosphate (cyclic GMP) is ineffective in influencing these responses to CD by HeLa.

**COLCEMID:** HeLa cells are induced to intense ruffling activity and filopodial extension by application of 0.1–0.6  $\mu\text{g/ml}$  of Colcemid. After some 30 min, the peripheral cytoplasm all about the cell

TABLE III  
Effect of Cyclic Nucleotides on CD-Induced Contraction and Zeiosis

	CD	CD + dbcAMP	CD + mbcGMP
% of initial area	60	81	51
% cells with zeiosis	79	7	46

HeLa monolayers were grown for 18 h in medium containing either 1 mM dibutyryl cyclic AMP and 1.2 mM theophylline, or 1 mM monobutyryl cyclic GMP. These, and DMSO controls, were then exposed to 0.1  $\mu\text{g/ml}$  of CD for 60 min. Area (occupied by 5 cells) was measured planimetrically and the percentage of 1,000 cells in each sample showing CD-induced zeiosis was scored. HeLa cells treated with dbcAMP + theophylline occupy two to three times as much area as untreated controls.

spreads out into very wide extenuated sheets (Fig. 11 *a, b*). Such cells, extended in 0.6  $\mu\text{g/ml}$  of Colcemid for 4–7 h, respond to the addition of 0.25–0.33  $\mu\text{g/ml}$  of CD by undergoing contracture, zeiosis and nuclear protrusion and clustering to the same extent and at the same rate as do control cells which had not been treated with Colcemid (Fig. 11 *c*), although some of the knobs are larger and misshapen. However, the central movement and aggregation of knobs and clusters to form crowns is impaired in Colcemid-treated HeLa, Vero, or HEp-2. The apical aggregates that develop at 1 or 2 h are usually loose and poorly massed, and clusters of knobs may remain dispersed on the topside of the cell separate from a major accumulation (Fig. 14). In these circumstances, peripheral sheets of cytoplasm are sometimes seen to become separated by clefts and to pull away from the main retracting cell mass.

The presence of Colcemid also interferes with recovery from the effects of CD. Many of the zeiotic knobs fail to recede even at one hour after withdrawal of the CD, and many nuclei remain protruded. Those nuclei that do reinsert in the presence of Colcemid remain eccentrically placed near the periphery, and fail to reoccupy the cell center as they do in the absence of Colcemid.

#### Differences in Responsiveness to CD among Various Cell Types

The incidence of zeiosis and of nuclear protrusion, in the cell types that exhibit these responses, is concentration dependent in a range from a

minimum to a limit which usually does not exceed 1  $\mu\text{g/ml}$ . This range, and thus the dose-response pattern of the various types, differ. Although contraction appears to be the initial observable reaction to effective concentrations of CD in all cell lines examined, zeiosis does not ensue in some lines, e.g., MDBK. HeLa, HEp2, and KB cells form a group responding similarly to CD with marked zeiosis at lowest concentration; Vero cells, L, and PR105, and XC fibroblasts, (like other lines of nonepithelial origin) are less sensitive by nearly an order of magnitude and project smaller and fewer knobs (Table IV). MDBK cells, although strongly contracted, are relatively resistant to zeiosis even at 2  $\mu\text{g/ml}$ .

Although nuclear protrusion is interpreted to be a variant of zeiosis, higher concentrations of CD are required to elicit this phenomenon. For the HeLa, KB, HEp2 group which express their nuclei reluctantly, the median concentration required to elicit nuclear protrusion is some 15–20 times that necessary to give zeiosis in one-half of the cells; whereas in Vero, PR105, L, and XC cells, which eject their nuclei very readily, only twice the median concentration of CD necessary for zeiosis,

TABLE IV  
Differences in Zeiotic Response to CD of Various Cell Types

Cell type	Median concentration* to effect:		
	Zeiosis	Nuclear protrusion	Cells crowned†
			%
HeLa	0.05	0.85	>85
HEp2	0.05	0.80	>90
KB	0.04	0.75	>90
Vero	0.35	0.75	<25
L	0.30	0.60	25
PR105	0.33	0.66	30
XC	0.25	0.5	50

\* Concentration of CD ( $\mu\text{g/ml}$ ) eliciting zeiosis or nuclear protrusion in 50% of cells after 60 min.

† Incidence of apical aggregation of zeiotic knobs (crowning) after exposure to 0.5  $\mu\text{g/ml}$  (HeLa, HEp2, KB), or 1.0  $\mu\text{g/ml}$  (all others) for 180 min.



is required (Table IV). MDBK cells, mouse macrophages, and human leukocytes are virtually resistant to nuclear extrusion at subtoxic doses.

The fate of the knobs on the free surface in the continued presence of CD, also differs in the different cell types. In the HeLa, KB, HEP2 group, early and tight clustering, and relatively speedy central translation and aggregation of clusters into large, compact apical crowns (bouquets, such as are depicted in Figs. 1, 4, 5, 12) occurs in almost all of the cells. In Vero cells, L, and fibroblast lines, the knobs, which are fewer and smaller, may cluster less compactly, and move centripetally in less organized array (Fig. 15), forming apical groupings in only some of the cells (Table IV). Whereas in HeLa, HEP2, and KB cells, the aggregated knobs are relatively stable structures in the presence of CD, in the fibroblast lines it appears that some of the small knobs initially protruded at the periphery in response to CD may subsequently recede, and that, although aggregation of knobs may begin in a higher proportion of cells during the first 30–60 min, many of the individual knobs or clusters are subsequently resorbed so that only some 20–50% of cells carry identifiable apical aggregates by 3 h, and these are obviously small. This disaggregation of clusters and retrocession of knobs probably corresponds in time with the much more prominent protrusion of nuclei in the latter lines.

## DISCUSSION

We have used the term zeiosis to designate the protrusion of blunt processes containing endoplasm at the cell surface in the form of knobs. This word was introduced by Costero and Pomerat (15) to describe the rapid projection and retrocession of blunt processes at the cell surface. Other observers have preferred to refer to essentially the same process as blebbing (28, 45, 54, 62). To the extent that the knobs evoked by CD remain protruded as relatively stable structures as long as CD is present, knobbing after CD differs from most other instances of zeiosis in which the protrusions are rapidly put forth and resorbed. However, the essential feature of zeiosis is herniation of endoplasmic components through the cortex, such as has evidently occurred in the examples depicted by Landau and McAlear (36), Rose (51, 52) and Price (46) *inter alia*. It is to be distinguished from blistering at the cell surface, sometimes called pycnotyosis (69) which is characterized by cortical

oedema or hydropic vacuolation. Zeiosis has been described in cultured interphase cells in a variety of circumstances: physiological (9, 15, 51, 5, 28, 27); experimental (36, 51, 48, *inter alia*); and pathological (8); it is a regular feature of anaphase (9), of normal G<sub>1</sub> cells in subconfluent monolayers, and it may occur throughout the cell cycle in isolated cells (45, 54). Zeiosis or blebbing has been well described during normal development and cell movement *in vivo*, and the roles of such surface protrusions (blebs) in the formation of cellular organs of locomotion has been underscored by Trinkaus (62). Cells of epithelial derivation exhibit spontaneous zeiosis much more readily than other cell types (28, 48), which accords with the greater sensitivity of HeLa, HEP2, and KB cells to CD (Table IV).

Increased intracellular pressure as well as the occurrence of "gaps" in the cortex, upon both of which extrusion of some endoplasm appears to depend, are the apparent consequences of the generalized cell contraction induced by CD. This is manifested in the cortex by a condensation and displacement of subplasmalemmal microfilaments which leaves sectors of the cell membrane largely denuded of the normal microfilament web (39). The upper surface of the flattened marginal velum of the extended cell is evidently the most vulnerable zone, and the knobs first erupt here after CD as well as in spontaneous zeiosis (28). The membrane required to cover the many protrusions is presumably derived from withdrawal of microvilli (38), and the rounding up of the cell. The endoplasm must be sufficiently fluid to permit its expression; agents that affect the tonicity of the medium and consequently of the cytoplasm such as sorbitol (28), prevent zeiosis by making the endoplasm more viscous, probably reducing the actual internal hydrostatic pressure (18).

The protuberances in physiological zeiosis are often more sessile and broadly based than the knobs elevated on tall slender stalks characteristic of CD (Fig. 6). These stalks maintain an erect and contracted form; microtubules are very rarely present in them, nor does colchicine prevent their elevation. In the zeiotic processes described by Rose (52), constriction rings were seen to move distad in peristaltic waves. But in the presence of CD, the microfilament-containing stalk remains fixed in contracture. Compacted microfilament masses, which underlie the knobs and migrate with them, also obturate their proximal peduncles (Figs. 5–8) and may constitute a barrier between

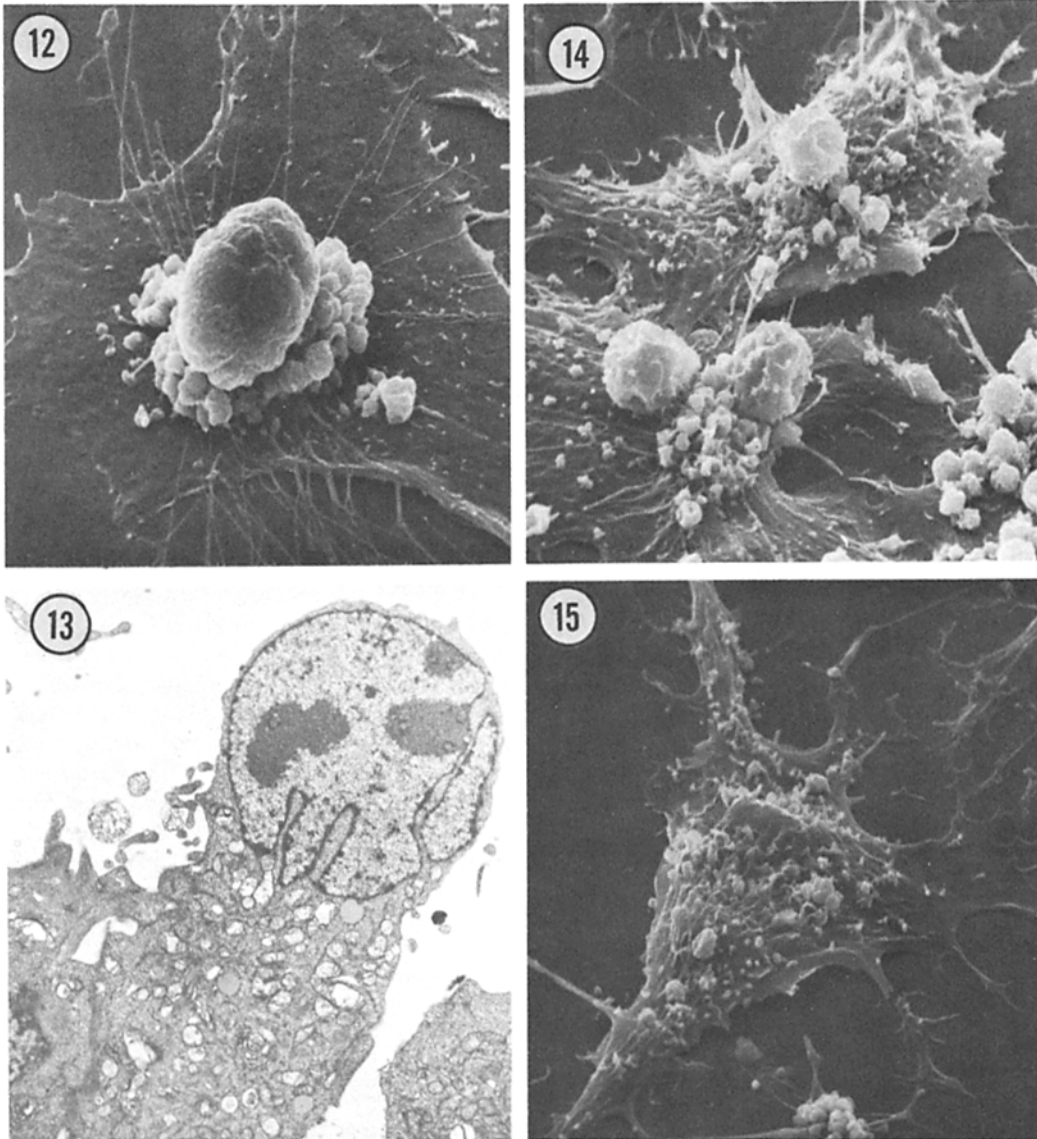


FIGURE 12 Scanning electron micrograph of a HeLa cell after 4 h of exposure to 0.25  $\mu\text{g}/\text{ml}$  CD. The nucleus protrudes at the apex, presenting a smooth surface (cf. Fig. 11). It is surrounded at its base by a tight collar of aggregated knobs. Retraction fibers radiate from the margins of this collar across the surface to make adhesive contact with substrate, or with neighboring cells. Remnants of microvilli are sparsely distributed on the surface.  $\times 2,200$ .

FIGURE 13 Section through a HeLa cell after exposure to 5  $\mu\text{g}/\text{ml}$  of CD for 3 h. The protruded nucleus is covered by a thin envelope of cytoplasm. The incisures in the nuclear profile are sections through convolutions resulting from passive compression of the nucleus during its herniation. This high dose of CD is somewhat toxic, as manifested by the mitochondrial swelling. Note the very straight profile of the margin of the adjacent cell.  $\times 6,000$ .

FIGURE 14 HEp2 cells treated with Colcemid (0.6  $\mu\text{g}/\text{ml}$ ) for 7.5 h, followed by exposure for 1 h to 0.25  $\mu\text{g}/\text{ml}$  of CD, in the presence of Colcemid. The aggregates of knobs are not as tightly massed (cf. Figs. 4 d and 12), and some clusters remain dispersed.  $\times 2,200$ .

FIGURE 15 Scanning electron micrograph of a Vero cell after exposure to CD (1  $\mu\text{g}/\text{ml}$ ) for 1 h. The top surface of the retracted, arborized cell exhibits stubby remnants of withdrawn microvilli. The nucleus bulges outward at the center. Compare with the distribution of knobs in comparably treated HeLa in Figs. 4 and 12.  $\times 2,200$ .

the contents of the distal knobs or bulk and the main cell body.

At 1 h, overall incorporation of precursor into protein is reduced by about 20% in the whole CD-treated cell. CB produces an identical diminution in fibroblasts (A. Springer, and J. Perdue, cited in reference 11) but apparently none in some other cell types (11). The overall decrease may reflect the greater diminution of protein synthesis in the knobs as compared with the residual cytoplasm in the main body of the cell. This difference conforms with the morphology of the endoplasm which, in the clavate knobs, consists mainly of concentrated monoribosomes (or subunits), and in the cell body contains polyribosomes and rough endoplasmic reticulum and but very few monoribosomes. Although there are polyribosome clusters in the endoplasm of the nascent protuberance, they probably disaggregate in the distal expansions of the knobs, where they may be sequestered from those products of the nucleus necessary for initiation of protein synthesis (44).

Expulsion of endoplasmic organelles (mitochondria, rough endoplasmic reticulum, lysosomes) into the protuberances has been noted in zeiosis occurring in various circumstances (46, 52, 36). Among the organelles sometimes ejected, as in the zeiosis of cells growing under nitrocellulose membranes, are nuclei (52), and the enlarged protrusions containing nuclei may sometimes become detached, resulting in "autoenucleation" of the cells (52). These events obviously occur much more frequently and regularly, but with the differences mentioned, in cells exposed to CD. Expression of the nucleus from the cell center and its protrusion at the surface in a distended envelope of cytoplasm under the influence of CD can thus be regarded as a special form of zeiotic herniation of endoplasmic contents. It is not clear why nuclear protrusion and subsequent enucleation should occur so much more readily after cytochalasin treatment of nonepithelial lines (PR105, L, and XC cells; and Vero) than in the cells of epithelial derivation (HeLa, Hep2 and KB), in which zeiosis is more prominent; or why MDBK, although contracted, hardly exhibits either. Chinese hamster ovary cells in  $G_1$  actually lose their numerous and prominent surface bosses when exposed to CB, but small zeiotic knobs do erupt at the periphery (23). The reason for these variations is probably to be found in differences in the organization of the cortex in different cell types, and perhaps in the consequent

differences in size of the undefended subplasmalemmal gaps that develop soon after exposure to CD. In the epithelial cell lines, in which nuclear protrusion is less prominent and more membrane therefore available to cover, the knobs are generally larger and more numerous, whereas the converse is true of fibroblastic and other nonepithelial derivatives.

The presumed augmentation of intracellular tension and redistribution of the cortical microfilaments (38) concomitant with CD-induced contraction of ectoplasm are prevented by inhibitors of energy metabolism (38), and zeiosis is also therefore inhibited by these agents (Table II). Increased intracellular levels of cyclic AMP (supposedly by "stabilizing" the microfilament web or associated contractile elements) affects a number of activities of the cell surface which include ruffling, adhesion, contact, and locomotion (32, 33, 26, 30, 22, *inter alia*). Elevated cyclic AMP also causes inhibition of zeiosis occurring spontaneously (48), as well as that induced by CD (Table III). This information can perhaps be correlated with the higher levels of cyclic AMP in confluent nontransformed cells in density-dependent inhibition of growth (42, 43, 53, 56); these cells exhibit little spontaneous zeiosis after  $G_1$  (45, 54). This information can also correlate with the generally lower levels of cyclic AMP in cells in M and during some part of  $G_1$  (10, 68, 57) and presumably also in isolated growing cells; both of these types of cells exhibit a more marked tendency to spontaneous zeiosis (45, 54). Knobbing is much more readily evoked by lower concentrations of CD in the latter group (38). CB has been said not to affect the cyclic AMP level (35); it does not alter the ATP content of cells (64, 63).

#### *Movement of Zeiotic Knobs and of Markers at the Cell Surface*

The central migration of the knobs elicited by CD is similar in vector and rate to the rearward movement of ruffles (lamellipodia) on the dorsal surface of the normal attached cell (1, 31, 47, 28, 50). Indeed the zeiotic protrusions (blebs) that appear at the margins of cultured cells in physiologic conditions (as in respreading after subculturing) have been considered by Harris (28) to be ruffles swollen into blebs. Such transient blebs also gradually move inward on the top surface of the cell as they recede (28). Lamellipodia themselves are structurally very different from zeiotic protrusions.

sions: ruffles are essentially sheets of ectoplasm in which a network of thin microfilaments connects the inner aspects of the opposed lamellae of plasma membrane (3, 18, 47), whereas zeiotic bumps are protrusions of endoplasmic contents in which there are virtually no microfilaments. Microparticles picked up at the cell margin that adhere to the surface of untreated fibroblasts (2, 4, 28, 29) or cells of epithelial derivation (18) are also transported rearward like the ruffles. In fibroblasts marker particles move at rates between about 2–4  $\mu\text{m}/\text{min}$ , and they tend to accumulate on the upper surface, usually toward the rear (2, 29, 28, 4). On the surface of normal HeLa cells, although the vector of such attached particles tends generally inward, there is much lateral displacement, and they fail to accumulate in a large central cluster, at least within a few hours. But in colchicized HeLa, they rapidly and consistently move to the apex where they cluster (Fig. 11); a result which can be interpreted to imply that the mobility of particles attached to the HeLa cell membrane may be limited by colchicine-sensitive structures. This surface movement of particles, like that of the knobs, is clearly neither concomitant with nor dependent on translatory locomotion of the whole cell (28), and it can take place in the presence of immobilizing concentrations of CD (Fig. 10). Microparticles so conveyed are somehow adherent to the plasma membrane (29, 28, 6, 2), an attachment the tenacity of which may be dependent on a local reorganization of the underlying and adjacent microfilaments system, like that postulated by Allison (7) and demonstrated in macrophages by Reaven and Axline (49). The base of the cortical site underlying attached macromarkers appears in a conformation indicative of spreading or stretching (49), but the edges may be locally contracted (7, 66, 67). The nesting of particles between lips of membrane depicted by Albrecht-Buhler and Yarnell (6) also suggest this. These rearrangements are probably prefatory to phagocytosis. This cortical locus and the particles engaged on the overlying surface membrane probably move in the plane of the membrane as an integral unit. Transmembrane connection of surface binding sites with structures belonging to the inner aspect of plasma membrane has been shown for the erythrocyte (41), and can be presumed for cells with a contractile cortex. The failure of latex spherules to attach to HeLa cells after the clustering of zeiotic knobs in CD may result from the mobilization and displacement of much of the microfilament web of the cortex (39).

The peripheral surfaces of such a cell, much of which had thus become relatively denuded of microfilaments, would be incompetent to engage or to “nest” adsorbed microparticles.

The more stable knobs protruded under the influence of CD, and especially the small clusters of such knobs, may be analogous to attached particles in that their subjacent contracted microfilament feltworks together with the surmounting knobs of endoplasm are carried like particles, and both appear to move in the plane of the membrane as a unit or assembly.

The observation by Albrecht-Buhler and Solomon (5) that pretreatment with CB did not affect the small relative displacements (motion constant) of colloidal gold particles on the surface of 3T3 fibroblasts was interpreted to show that CB did not alter the viscosity of the membrane itself. Because aggregation and disaggregation of groupings of these particles would appear to have occurred with almost equal relative frequencies, no clusters were visualized on the surfaces of 3T3 cells (5). However, CB reportedly stops particle movement on the surface of epithelial cells (18).

The clustering of zeiotic knobs at the periphery, close to the margins from which they first erupt, and their subsequent centripetal movement and coherence in a single tight aggregate, is reminiscent of the formation first of “patches” and then of “caps” on many different kinds of cells by cell surface receptors (such as Ig, lectin-binding sites, histocompatibility and other surface antigens) bound or cross-linked by appropriate ligands (60, 17, 21, 14, 59, 33, 37, 40, 43, 20, *inter alia*). Like capping (63, 17, 21, 14, 13, 35, 62, 38, 41, 37, 40), aggregation of zeiotic knobs in CD is temperature dependent (Table I) and requires the provision of metabolic energy (Table II). Translatory cell locomotion is apparently as unnecessary for aggregation of knobs (or of macromarkers) as for patching and capping (55, 17). In many experiments the effects of CB on patching and capping have been described as partial or small (61, 17, 21, 34, *inter alia*). While such equivocal effects of CB on movements of membrane markers of lymphoid cells may result from the occurrence of two kinds of loci of capping, only one of which is inhibited by CB (59), at least as important a factor in such experiments may be the duration and completeness of action of cytochalasin. Inhibition of major centripetal or poleward membrane movements dependent on the microfilament apparatus can be expected only after the latter has been fully

contracted and displaced. Ukena et al. (63) observed that patches of bound concanavalin A, which in untreated SV3T3, are localized over the central part of the cell, were found all over the surface of cells pretreated with CB. These, however, did not cap. Colchicine, on the other hand, promoted central capping of the patches (63), as it does of microparticles on the HeLa cell surface (Fig. 11b). These experiments suggest that the major centripetal flow of the patch-bearing plasma membrane may be inhibited only after CB has been given an opportunity to exert a maximal effect.

Formation of small clusters of surface receptor by ligand (patching) appears to depend mostly upon undirected lateral movements in the fluid membrane, that can in some cases occur even at 4°C and may require no metabolic activity (24, 17). In this respect, patch formation appears to resemble the initial peripheral clustering of knobs (Table I). But cap formation, like the centripetal movement and aggregation of attached particles (and of CD-induced knobs), depends upon vectorial transposition of islands of membrane actively motivated by a temperature- and energy-dependent mechanism, provided that the mobility of such units is not restricted, as by colchicine-sensitive structures. This movement would appear to require participation of the cortical microfilaments. Aggregation might involve mutual engagement and interaction of the contracted microfilament components associated with and underlying knobs, attached microparticles, and perhaps also patches, the coalescence of which apparently produces the remarkable feltlike masses underlying the zeiotic crowns (Figs. 5-7) (39) and the dense filament networks subjacent to lymphocyte caps (17).

In the contraction induced by CD, much of the marginal zone of the cell, including zeiotic knobs and attachment fibers, is retracted centrally in an initial rather massive centripetal movement occurring mostly during the first 60-90 min of exposure to CD. This centripetal movement appears to require a microfilament system in the cortex capable of participating in contraction, and it therefore only occurs early after application of CD, before full contracture and displacement of the microfilament web. Although the microtubular system may normally provide anchorage for such surface structures as ligand-induced patches or attached particles (63, 19, *inter alia*), it would appear to offer them neither directional guidance

nor motive force. When these assemblies are released by microtubule-disruptive agents like colchicine, central aggregation of macromarkers (crowning, Fig. 11 *b, c*) or of patches (capping) (63, 19) is promoted.

By whatever means, membrane must ultimately be provided for the respreading (more accurately, flattening or "pseudospreading") of the peripheral cytoplasm of cells initially contracted by CD. This process, more prominent in some cell types than others, and from which cytochalasin derives its name, can be regarded as probably resulting from isodiametric flux (a "puddling down") of the more solated endoplasm uncontained at the periphery by a contractile cortex. The subplasmalemma of the flattened periphery of the cell that has respread while in CD is largely divested of microfilaments, most of which will have concentrated in the contracted apex in the form of the condensed feltworks like those depicted in reference 58 and in Figs. 5, 7.

#### Addendum

Since submission of this paper, K. Porter, T. Puck, A. Hsie, and D. Kelly (1974. An electron microscope study of the effects of dibutyryl cyclic AMP on chinese hamster ovary cells. *Cell*. 2: 145-162) have carefully documented the disappearance of surface knobs of cultured cells under the influence of dibutyryl cyclic AMP and the role of microtubules in the changes of cell form effected by this agent. M. R. Gershenbaum, J. Shay, and K. R. Porter (1974. Proceedings of a Workshop on Advances in Biomedical Applications of SEM. Part III) have demonstrated how the network of thin microfilaments is redistributed under influence of CB in whole mounts of cells in the high-voltage electron microscope.

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