

## A Permeabilized Cell Model for Studying Cytokinesis Using Mammalian Tissue Culture Cells

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**ABSTRACT** PtK<sub>1</sub> cells lysed late in cell division in a medium containing the nonionic detergent Brij 58 and polyethylene glycol will continue to undergo cleavage after lysis. Maintenance of cleavage after lysis is dependent on the composition of the lysis medium; the pH must be around neutrality, MgATP must be present, and the free Ca<sup>++</sup> concentration should be 1 μM for optimal constriction to occur. Cleavage can be stopped and reinitiated by raising and lowering the Ca<sup>++</sup> levels in the lysis medium. Cleavage in the permeabilized cell is blocked by addition of phalloidin, cytochalasin B, and *N*-ethylmaleimide-modified myosin subfragment-1 to the lysis medium. This represents the first cell model system for studying cleavage since the pioneering studies of Hoffman-Berling in 1954.

During cytokinesis in animal cells, forces are generated at or near the cell's surface which lead to the formation of the cleavage furrow and eventually to constriction of the cell in two. The organelle responsible for these events, the contractile ring, is a well-defined array of microfilaments located circumferentially in the cell beneath the plasma membrane of the concave portion of the cleavage furrow (1, 29, 33). Its existence is transient and coincides with the time of furrow formation and function (31). With immunological techniques and heavy meromyosin (HMM) decoration, actin, myosin,  $\alpha$ -actinin, and tropomyosin have been found in the cortex of the cell coincident with furrowing activity and may be concentrated in the contractile ring (3, 9, 10, 30, 32). The hypothesis that the contractile ring is directly responsible for generating the forces required for cleavage is supported by considerable physiological evidence. Inhibitors of furrowing, such as high pressure, and drugs, such as cytochalasin B, disrupt contractile ring organization (33, 38, 40). Mechanical disruption of the cortex where the ring is located prevents tension generation and leads to failure of cleavage (28). In addition antibodies against myosin injected into starfish eggs and *N*-ethylmaleimide (NEM)-modified HMM microinjected into amphibian eggs block cleavage (17, 20, 23).

Although it has been suggested that the contractile ring operates in some ways analogous to a muscle sarcomere (1, 29, 33), it must be recognized that there are significant differences in the way the contractile ring functions. The organization of actin in the contractile ring is quite different from that in muscle and, unlike a sarcomere, the contractile ring has a transient existence (31). It is organized in the cortex during

telophase and progressively disassembles as cleavage proceeds. At present, no information is available concerning the process of assembly and disassembly of the contractile ring and relatively little is known about the organization of the mechanical elements in the furrow.

Our ignorance, in part, is because of the lack of cell models for studying cytokinesis. With the exception of the pioneering work of Hoffman-Berling (15, 16) and Hoffman-Berling and Kinoshita (18), there have been no reports of model systems for studying cleavage. Hoffman-Berling (15, 16) demonstrated that after a brief glycerination, dividing fibroblasts would undergo cleavage in the presence of MgATP at a slightly alkaline pH. Unfortunately, these experiments were not performed in a manner that allows one to estimate the calcium requirements for cleavage. Although glycerol has been used extensively since Hoffman-Berling's time for the preparation of cytoskeletons for HMM decoration (11, 30, 32) and to study fibroblast movement in culture (11), the glycerinated cell preparations have not been used successfully to study cleavage in vitro. Given the importance of cleavage in cell division and our lack of knowledge about how this and other motile processes are regulated in nonmuscle cells, it would be useful to extend Hoffman-Berling's studies and to reestablish a permeabilized model system for investigating the mechanism of furrow constriction.

I report here the use of a nonionic detergent lysis protocol to prepare cell models that undergo cleavage after lysis. The Brij 58-polyethylene glycol lysis procedure is similar to the one previously described (5), which maintains anaphase chromosome movement in permeabilized PtK<sub>1</sub> cells. After lysis, the

plasma membrane, mitochondria, and other membrane-bounded organelles are partially disrupted but the ground substance and the cytoskeletons of these cells appear to be similar to those of unlysed cells (Cande, Meeusen, and McDonald, manuscript submitted for publication). Maintenance of cleavage after lysis is dependent on the composition of the lysis medium, particularly the pH, levels of free calcium ions, and levels of MgATP present in the medium. By manipulating these parameters, it is possible to stop and restart cleavage. I have used the permeabilized cell model to describe the optimal pH and free calcium levels required for cleavage after lysis and to describe the effects of various drugs and proteins, such as cytochalasin B, phalloidin, and NEM-modified myosin subfragment-1 (NEM-S<sub>1</sub>), on cleavage.

## MATERIALS AND METHODS

### Materials

Vanadate-free ATP prepared from yeast was obtained from Boehringer-Mannheim Biochemicals, Indianapolis, Ind. Carbowax 20 M (polyethylene glycol, 20,000 mol wt), cytochalasin B, and Brij 58 were obtained from Sigma Chemical Co., St. Louis, Mo. To reduce variability in permeabilization experiments, stock solutions were stored frozen until the day of use and then discarded at the end of the day. I found that it was not possible to store aqueous Brij 58 stock solutions because the properties of the detergent changed with age. These solutions were made fresh each day. Phalloidin was a gift from T. Wieland (Max Planck Institute für Chemie, Heidelberg, W. Germany).

S<sub>1</sub> was prepared from rabbit skeletal myosin by the method of Weeds and Taylor (41). NEM-S<sub>1</sub> was prepared by the same method used previously to prepare NEM-HMM (22, 24). S<sub>1</sub> at 5–10 mg/ml was incubated in 10 mM imidazole-Cl, 0.2 mM dithiothreitol (DTT) with 1 mM freshly dissolved NEM, pH 7.0, at room temperature for 75 min. The reaction was stopped by adding DTT to a final concentration of 10 mM, and unreacted NEM was removed by exhaustive dialysis. ATPase activities of the treated proteins were measured as described previously (24). As with NEM-HMM, the calcium ATPase activity of NEM-S<sub>1</sub> was elevated above that of S<sub>1</sub>, and the actin-activated and K<sup>+</sup>/EDTA-stimulated ATPase activity of NEM-S<sub>1</sub> was <10% that of the untreated proteins. NEM-modified bovine serum albumin (NEM-BSA) was prepared in the same manner as NEM-S<sub>1</sub>.

Precise levels of free calcium in the lysis medium were maintained with a Ca/EGTA buffering system (Table I) designed to compensate for the presence of 1 mM excess Mg<sup>2+</sup> and 1 mM MgATP at a defined pH (27). These calculations were performed by R. Steinhart as described previously (43). The calcium chloride stock solution was obtained from Orion Corp., Cambridge, Mass.

### Tissue Culture Cells and Cell Lysis

PtK<sub>1</sub> cells were used in all experiments and were maintained and handled for light microscopy as described previously (5). Coverslips were mounted on slides with coverslip fragments as spacers. Cells engaged in cell division were lysed by flushing solutions under the coverslip in a two-step lysis procedure. Step A medium contained 85 mM PIPES, pH 6.94, or buffers specified in the text, 0.08% Brij 58, Ca/EGTA buffers, 1 mM ATP, 2 mM MgSO<sub>4</sub>. Step B, which followed 70–90 s after step A, used a similar medium that also included 2.5% carbowax 20 M and 0.1–0.15% Brij 58. For the pH experiments, the following buffers were

used in addition to PIPES: 85 mM MES [2-(*N*-morpholino)ethane sulfonic acid] at pH 6.4, 85 mM HEPES at pH 7.5, and 85 mM EPPS (4-[2-hydroxyethyl]-1-piperazine propane sulfonic acid) at pH 8.0. The chambers held ~40  $\lambda$  of lysis medium. At each step, 200  $\lambda$  (5  $\times$  volume) of medium were flushed through the chamber during a 20-s exchange period using bibulous paper to facilitate flow. All experiments were run at 35°  $\pm$  2°C and the temperature of the stage was maintained with a hairdryer (Lady Schick Tote 'N Dry, Schick Inc., Lancaster, Penn.).

### Light Microscopy

Films of dividing cells were made with Zeiss phase or Nomarski optics and an Opti Quip 16-mm ciné time-lapse apparatus (Opti Quip Inc., Highland Mills, N. Y.). Exposures of 0.2–0.8-s duration were made at a rate of 20 frames/min on Kodak RAR 2496 or 2498 film. A Xenon 150-W lamp (Kneisley Electric Co., Toledo, Ohio) was used as a light source with heat cut and 546-nm wide pass band interference filters.

## RESULTS

### Cleavage in PtK<sub>1</sub> Cells

Selected photographs taken from a ciné record of a normal PtK<sub>1</sub> cell undergoing cleavage are shown in Fig. 1A–E. Although these cells do not round up to the same extent as other cells in culture, PtK<sub>1</sub> cells thicken during the onset of cell division and assume a rectangular shape during anaphase and telophase. Furrowing begins after spindle elongation is complete. If the upper surface of the cell is monitored with a  $\times$  40 phase lens without altering the focal plane during cleavage, the center of the cell usually drops out of the plane of focus (Fig. 1D–E) before the cell margin is completely pinched in. The spindle midbody is not normally visible in the cleavage zone until constriction has progressed to near its end point. In unlysed cells, constriction (the change in width across the cell midzone) proceeds at a rate of ~1  $\mu$ m/min (Fig. 2). Because anaphase cells have an average width of 12  $\mu$ m, cleavage normally continues for 10–15 min before the cells are completely constricted.

### Cleavage in Lysed Cells

Cleavage in lysed cells (Fig. 1F–J) proceeds for 5–7 min in a manner similar to that observed in unlysed cells (Fig. 1A–E). After addition of lysis medium, constriction initially proceeds for 1–2 min at a rate faster than that observed in vivo and then decreases to ~0.5–0.75  $\mu$ m/min (Fig. 2). Normally, furrow diameters decreased by ~50% after lysis, but in some experiments constriction proceeded to <10% of the original furrow diameter. This represents a change in furrow diameter of 4–8  $\mu$ m. The extent of the zone of cytoplasm involved in furrowing showed considerable variation and ranged from 3 to 8  $\mu$ m.

After lysis, all dividing cells undergo some noncleavage-related shape changes (Figs. 3 and 4). In general, the rectangular-shaped telophase cells are reduced in length and, except for the furrow, increase slightly in width. This is similar to the shape changes reported by Goldman et al. (11) for fibroblasts after glycerination and addition of MgATP. The largest isotropic shape changes in telophase cells are observed in cells lysed early in telophase that have not yet begun to cleave (Figs. 3 and 4).

The occurrence of cleavage after lysis is dependent on the stage at which the cells are lysed (Figs. 3 and 4). Cells early in telophase that have no detectable furrow fail to undergo further cleavage after lysis (Figs. 3A and B and 4). Cells that have undergone cleavage for at least 2–3 min and have well-developed furrows undergo maximal cleavage after lysis (Figs. 3E–

TABLE I  
Recipes for the Ca-EGTA Buffer System

pH	[Mg <sup>2+</sup> ] added	[Ca <sup>2+</sup> ] added	EGTA added	Estimated free Ca <sup>2+</sup>
	mM	M	mM	M
6.4	1.03	4.55 $\times$ 10 <sup>-5</sup>	3	10 <sup>-7</sup>
7.5	1.05	6.66 $\times$ 10 <sup>-4</sup>	1	10 <sup>-7</sup>
8.0	1.04	9.15 $\times$ 10 <sup>-4</sup>	1	10 <sup>-7</sup>
6.94	1.03	1.75 $\times$ 10 <sup>-5</sup>	1	10 <sup>-8</sup>
6.94	1.03	1.51 $\times$ 10 <sup>-4</sup>	1	10 <sup>-7</sup>
6.94	1.01	6.41 $\times$ 10 <sup>-4</sup>	1	10 <sup>-6</sup>
6.94	1.00	8.81 $\times$ 10 <sup>-4</sup>	1	4 $\times$ 10 <sup>-6</sup>
6.94	1.00	9.57 $\times$ 10 <sup>-4</sup>	1	10 <sup>-5</sup>

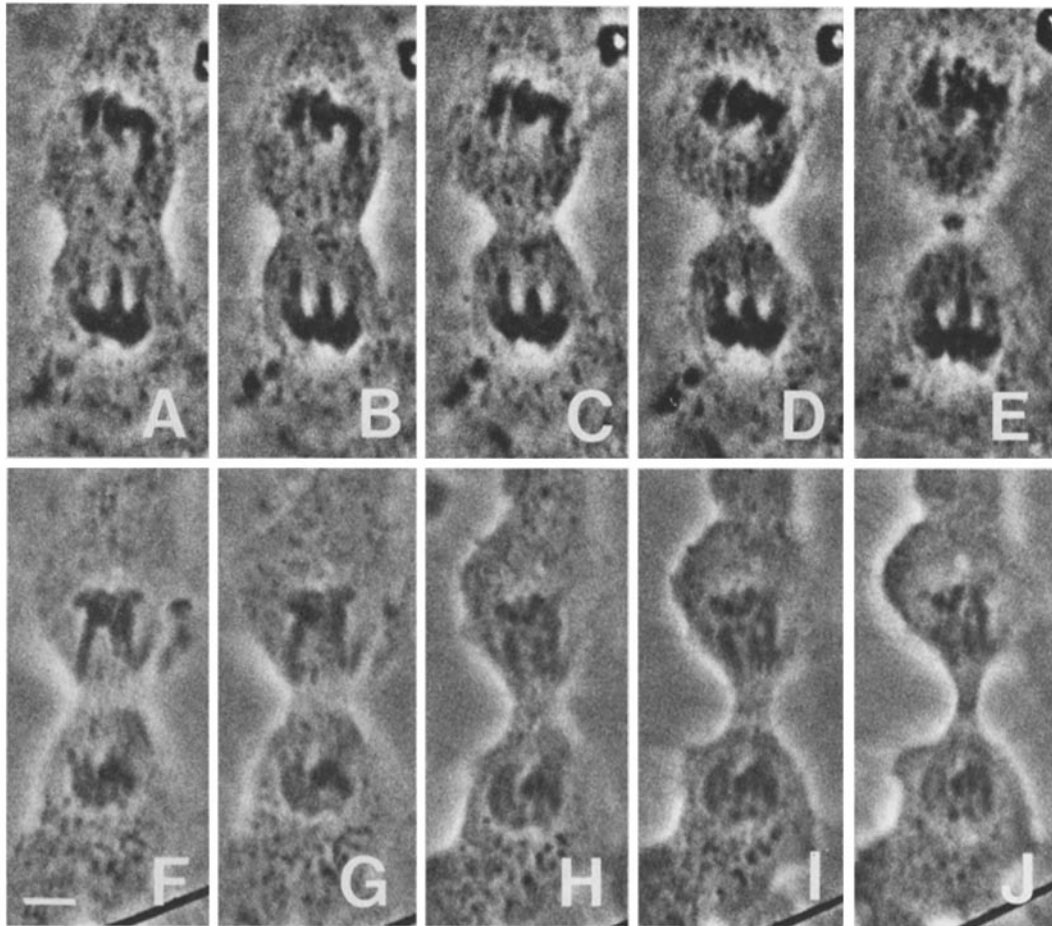


FIGURE 1 Selected frames from a ciné record of cleavage in an unlysed and lysed PtK<sub>1</sub> cell. A-E is a series taken of an unlysed cell in culture medium. A, 0 min; B, 1 min; C, 2.5 min; D, 4 min; and E, 10 min. F-J is a series of a cell lysed after 0.1 min in solution A containing 4  $\mu\text{M}$  free calcium and no MgATP. When solution B containing 0.1  $\mu\text{M}$  free calcium and 1 mM MgATP was added at 1.5 min, cleavage resumed. F, 0 min; G, 1 min; H, 2 min; I, 3 min; and J, 6 min. Bar, 5  $\mu\text{m}$ .

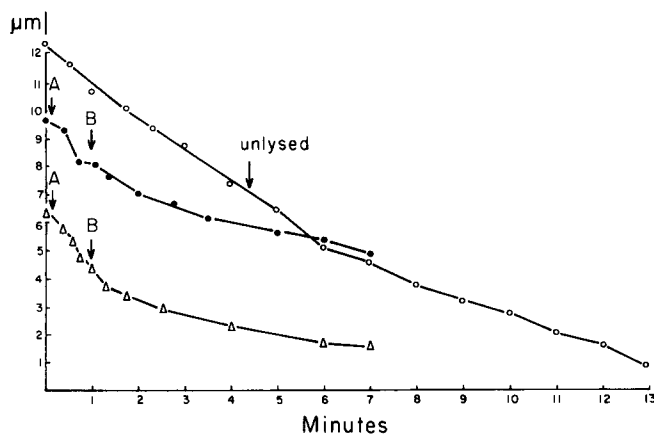


FIGURE 2 Change in furrow diameter in unlysed and lysed PtK<sub>1</sub> cells. Furrow diameters were measured from projected images of a ciné record of cleaving cells. A and B and the arrows indicate where solutions A and B were added during the lysis protocol. In these experiments, A and B contained 0.1  $\mu\text{M}$  free calcium and 1 mM MgATP.  $\circ$ , Unlysed cell;  $\bullet$ , cell lysed 1-2 min after initiation of cleavage;  $\Delta$ , cell lysed 3-4 min after initiation of cleavage.

H and 4). However, the response to lysis is highly variable in cells that have entered cytokinesis within the preceding 1-2 min and have small furrows (Figs. 3 C and D and 4). Of the five cells lysed at this stage, two cells contracted to 50% of their

original furrow width and the other three cells showed little constriction after lysis. In most experiments, cells equivalent to those in Fig. 3 E-H were used for experimental manipulation (for example see Figs. 1 and 2).

### Calcium and Cleavage

Ca/EGTA buffer systems, as described in Table I, were used to study the calcium requirements for cleavage. The optimal calcium concentration for maintenance of cleavage after lysis is near 0.1  $\mu\text{M}$  (Fig. 5). At concentrations  $>1 \mu\text{M}$ , further cleavage after lysis is blocked and the furrow slowly increases in width (Fig. 6). The effects of micromolar free calcium are not limited to just the furrow. Under these conditions, the margins of the cell after lysis become more indistinct and the cell does not display the isotropic shape changes seen at lower calcium concentrations (Fig. 10 A and B). Cleavage continues after lysis in  $10^{-8}$  M calcium or in 5 mM EGTA but furrowing is not so extensive as in 0.1  $\mu\text{M}$  free calcium. In 5 mM EGTA, the extent of constriction is only about one-half that observed in 0.1  $\mu\text{M}$  free calcium.

By manipulating the calcium and MgATP levels after lysis, it is possible to stop and restart cleavage (Fig. 7). Lysis in 4-8  $\mu\text{M}$  calcium in the absence of MgATP can be used to temporarily stop cleavage. At these calcium concentrations, furrow relaxation occurs more slowly in the absence than in the presence of nucleotide. The addition of a second solution

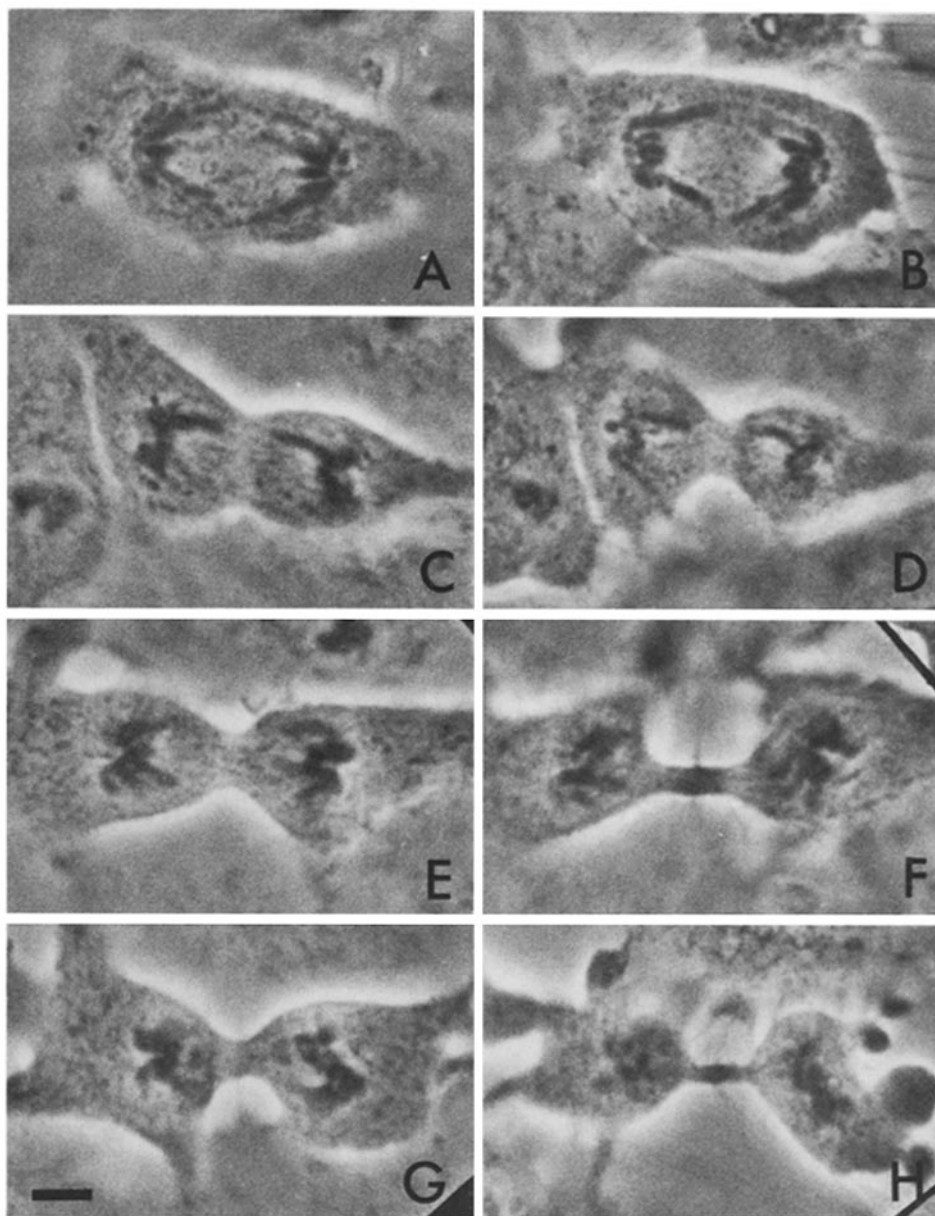


FIGURE 3 Cleavage in permeabilized cells lysed at different times after cleavage initiation. A, C, E, and G are phase micrographs of cells just before addition of lysis medium; B, D, F, and H are the same cells, respectively, 5 min after addition of lysis medium containing  $0.1 \mu\text{M}$  free calcium and  $1 \text{ mM}$  MgATP. In A, the cell is in early telophase and no furrow is apparent before or after lysis. In C, the furrow was initiated 1–2 min before lysis. In E, the furrow was initiated  $\sim 3$ –4 min before lysis and in G, 7–8 min before lysis. Bar,  $5 \mu\text{m}$ .

containing  $0.1 \mu\text{M}$  free calcium and MgATP will reinitiate cleavage, provided the exposure to suboptimal conditions after lysis is  $< 5$  min (Fig. 7). Micrographs taken from a film record of typical stop-restart experiments are presented in Figs. 1 F–J and 13 A–E. In these experiments, cleavage is retarded for 1.5 min before it is reinitiated by the addition of  $0.1 \mu\text{M}$  calcium and MgATP. Constriction is often not so extensive with the stop-restart protocol but reduction in furrow diameter of at least 30% is routinely observed.

### pH and Cleavage

I have studied the pH optimum for cleavage under conditions in which the free calcium levels were kept constant at  $0.1 \mu\text{M}$  (Table I) and the MgATP levels maintained at  $1 \text{ mM}$ . Under

these conditions, constriction proceeds to its farthest extent at a neutral pH (Fig. 8). At pH 8, the furrow diameter expands and margins of the cell relax in a manner reminiscent of lysis in free calcium levels  $> 4 \mu\text{M}$  (Figs. 6 and 10 C and D). Preliminary observations demonstrate that cleavage can also be stopped and reinitiated after lysis by manipulating the pH of the lysis medium. At pH 8, the isotropic shape changes normally observed after lysis are also inhibited (Fig. 10 C and D).

### Nucleotides and Cleavage

After lysis, maximal constriction occurs in the presence of  $5 \text{ mM}$  MgATP (Fig. 9). Some cleavage occurs in the absence of exogenous nucleotides, but this is less than one-half the con-

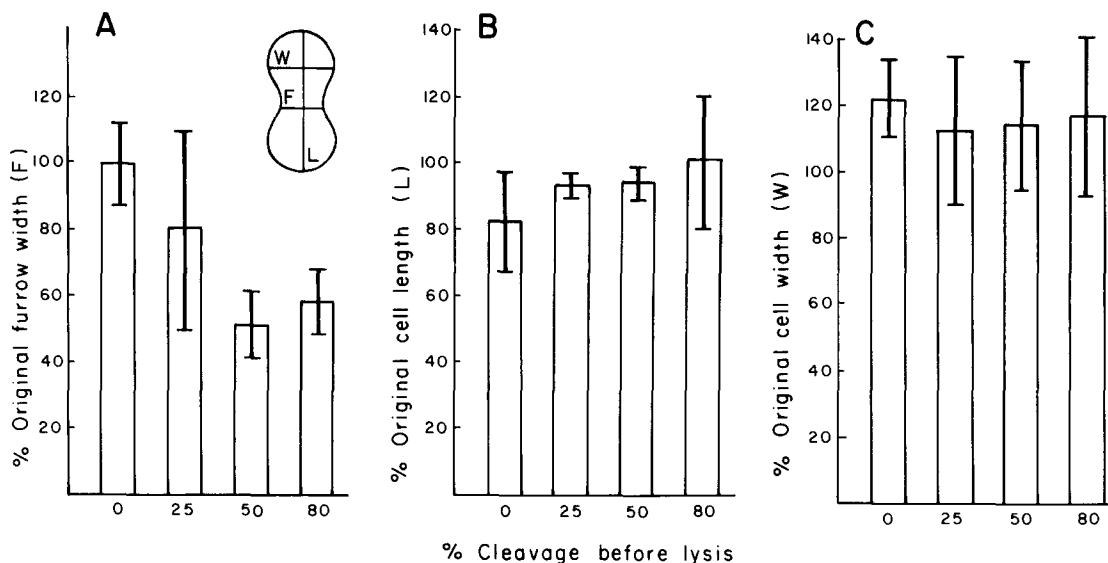


FIGURE 4 Extent of cell shape change in permeabilized cells lysed at different times after cleavage initiation in the unlysed cell. 0% cleavage is equivalent to the cell in Fig. 3 A, 25% cleavage, to the cell in Fig. 3 C, 50% cleavage, to Fig. 3 E, and 80% cleavage, to the cell in Fig. 3 G. (A) Furrow width (F) was measured at the point of maximum constriction. (B) Cell length (L) was measured as a straight line drawn perpendicularly through the centers of the two nuclei. (C) Cell width (W) was a measure of the cell diameter taken across one of the two nuclei. Each measurement is an average of five separate experiments. The error bars indicate standard deviation.

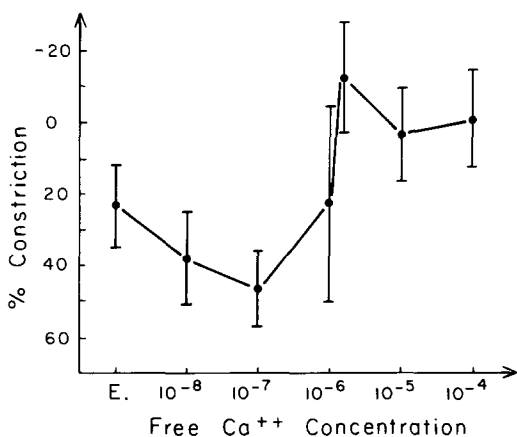


FIGURE 5 Extent of change in furrow diameter after lysis in different levels of free calcium ions. Percent constriction is defined as the change in furrow diameter divided by the original furrow diameter  $\times 100$ . The calcium levels were calculated as described in Table I; E is a solution containing 5 mM EGTA, 1 mM excess Mg, and no calcium. EGTA buffers were not used in experiments requiring the addition of  $10^{-4}$  M calcium. The error bars indicate standard deviation. Each point is an average of a minimum of five separate experiments.

striction seen in the presence of 5 mM MgATP. Presumably, there are intracellular stores of MgATP present in the permeabilized cell (5) which can be used to maintain some furrowing activity after lysis even in the absence of exogenous nucleotide. At high concentrations of MgATP, constriction is blocked. The increase in furrow diameter after lysis in 10 mM MgATP is less extensive and occurs less rapidly than the increases in furrow diameter observed when cells are exposed to alkaline pH or 4–10  $\mu$ M free calcium (Fig. 10 E and F). ITP will substitute for high ATP to relax the furrow and retard constriction but cleavage will still occur in 10 mM CTP and GTP (Fig. 9). Reduction of the ionic strength of the lysis medium to

compensate for addition of 10 mM Mg nucleotide does not alter the outcome of the experiment.

### Cytochalasin B

Cytochalasin B has an immediate and dramatic effect on the progress of cleavage in the permeabilized cell (Figs. 11 and 12 A and B). After lysis in 15  $\mu$ g/ml cytochalasin B, the furrow stopped contracting and within 1 min after lysis the furrow expanded to several times its width before lysis (Fig. 11). A concentration of cytochalasin B 30 times lower (0.5  $\mu$ g/ml) also led to furrow relaxation, but constriction continued for 1 min before it was blocked. Cells lysed in 0.1  $\mu$ g/ml cytochalasin B

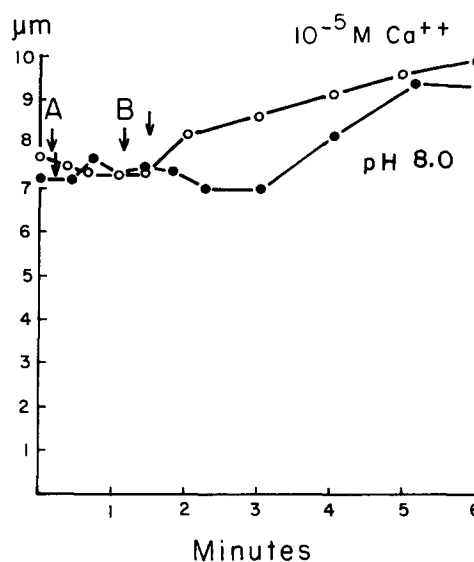


FIGURE 6 Relaxation of furrow diameter after lysis in high free calcium or alkaline pH. Cells were lysed in solution A and B in either 10  $\mu$ M free calcium at pH 6.94 (O) or in 0.1  $\mu$ M free calcium at pH 8.0 (●).

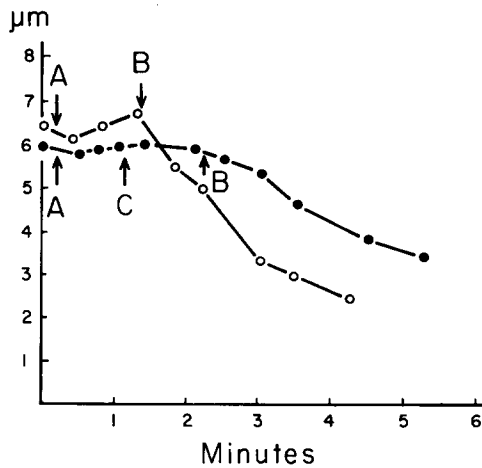


FIGURE 7 Delay and reinitiation of cleavage after lysis. Cells were lysed in solution A containing  $4 \mu\text{M}$  free calcium and no MgATP. Furrow diameter does not decrease until solution B, which contains  $1 \text{ mM}$  MgATP and  $0.1 \mu\text{M}$  free calcium, is added (O). In one experiment (●), solution C containing polyethylene glycol, no ATP, and  $4 \mu\text{M}$  free calcium was added. Furrowing was delayed for an additional 1.5 min before solution B was added.

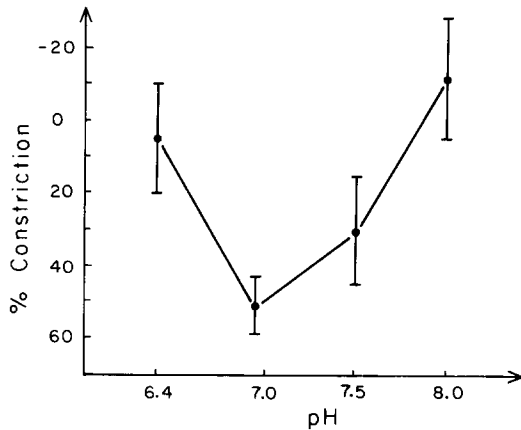


FIGURE 8 Extent of change in furrow diameter after lysis in solutions of different pH. All solutions contained  $0.1 \mu\text{M}$  free calcium as described in Table I. The error bars indicate standard deviation.

still undergo cleavage after lysis, but both extent and rate of constriction are slightly reduced compared with dimethyl sulfoxide controls.

For comparison, cells in culture medium were also treated with  $15 \mu\text{g/ml}$  cytochalasin B. After drug addition, furrowing ceased within 2 min. The cleavage furrow began to relax and, after 5 min, the uncleaved shape of the early telophase cell was restored. Unlike the situation in permeabilized cell models, the margins of the cell did not balloon out. This cell, when lysed into a drug-free medium, did not undergo cleavage (W. Cande, unpublished data).

### Phalloidin

The addition of  $200 \mu\text{g/ml}$  phalloidin to the culture medium had no effect on the progress of cytokinesis in unlysed cells. By manipulating the free calcium and MgATP levels in the lysis medium, cleavage after lysis was delayed for 90 s in the presence of  $200 \mu\text{g/ml}$  phalloidin (Fig. 11). Under these circumstances, phalloidin blocked reinitiation of cleavage (Figs. 11 and 12 C and D). Although the margins of the cell in the

furrow were deformed slightly after MgATP and  $0.1 \mu\text{M}$  calcium were added to the medium, little or no constriction occurred in the presence of the drug. The furrow did not relax or increase in width after drug treatment but the cells did undergo some isotropic shape changes (Fig. 12 C and D).

### NEM-S<sub>1</sub>

As has been described previously for NEM-HMM (22, 24), NEM-S<sub>1</sub> inhibits super precipitation of actomyosin solutions, the contraction of glycerinated myofibrils in the presence of MgATP, and the contraction of strands of *Chaos* cytoplasm. NEM-S<sub>1</sub> at a concentration of 1–2 mg/ml completely blocks reinitiation of cleavage in permeabilized cells after a 90-s incubation in the protein (Figs. 13 F–J and 14). In the presence of NEM-S<sub>1</sub>, the furrow gradually relaxes and increases in width, suggesting that NEM-S<sub>1</sub> interferes with the structural integrity of the furrow. NEM-S<sub>1</sub> retards cleavage after lysis even if the stop-restart protocol is not used, but the permeabilized cell continues to cleave for 1–2 min before cleavage is inhibited. NEM-BSA and S<sub>1</sub> have no effect on the rate or extent of constriction in the permeabilized cell (Figs. 13 A–E and 14).

### DISCUSSION

By manipulating the lysis medium, I have been able to maintain, stop, and reinitiate cleavage in permeabilized mammalian cells. This is the first model system for studying cleavage since the pioneering studies of Hoffman-Berling (15, 16), Hoffman-Berling and Kinoshita (18), and Arronet (2) using glycerinated fibroblasts. After lysis, two types of contraction are observed in the permeabilized telophase cell. First, cells round up after lysis. This isotropic contraction of the cytoplasm of the rectangular telophase cell results in a decrease in cell length of 10–20% and an increase in overall cell width of 10%. Second, in cleaving cells, furrow constriction continues at almost in vivo rates after lysis, leading to a decrease in furrow diameter of 50%.

Constriction after lysis is observed in all cells that have established a furrow and is not observed at all in early telophase cells. The greatest variability in extent of constriction after lysis

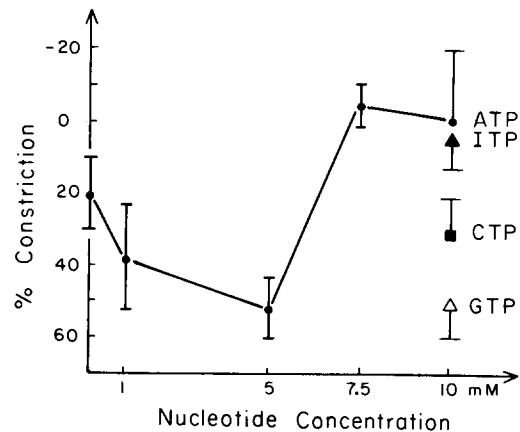


FIGURE 9 Extent of change in furrow diameter after lysis in solutions of different nucleotide composition. All solutions contained  $0.1 \mu\text{M}$  free calcium, the appropriate levels of Mg nucleotide, and  $1 \text{ mM}$  excess Mg. For experiments where  $7.5 \text{ mM}$  or  $10 \text{ mM}$  nucleotide was added, the PIPES buffer was reduced to 50 and 35 mM, respectively. The error bars indicate standard deviation.

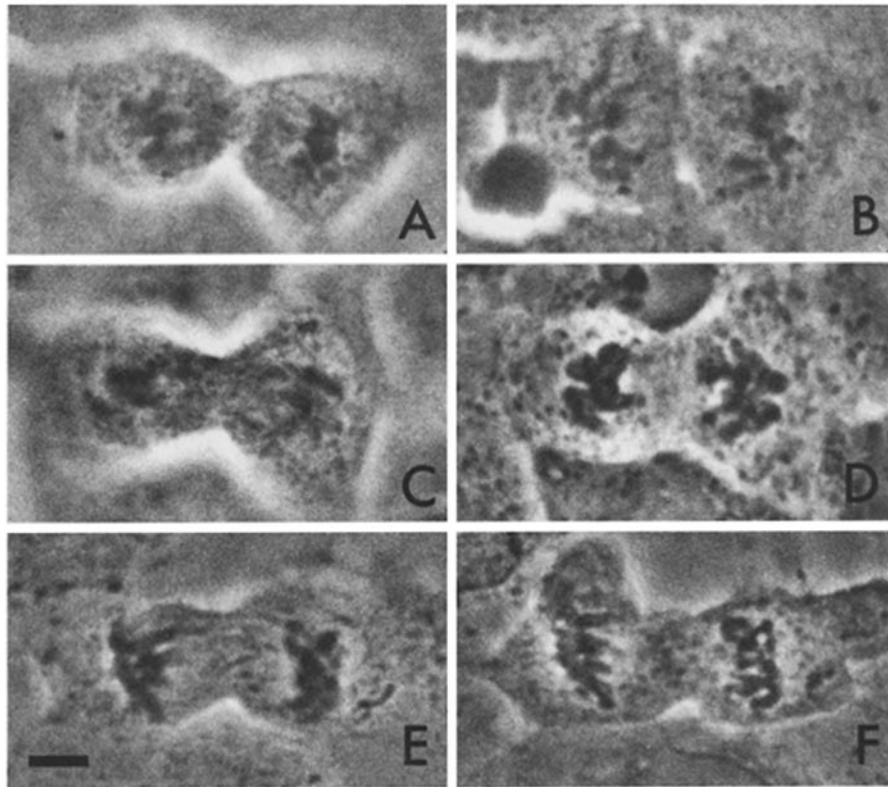


FIGURE 10 Cleavage in different physiological conditions. A, C, and E are phase micrographs of cells just before addition of lysis medium; B, D, and F are the same cells, respectively, 5 min after lysis. In A, the cell was lysed in medium containing  $10 \mu\text{M}$  free calcium at pH 6.9, in C, the medium was at pH 8, and in E, the lysis medium contained 10 mM ATP at pH 6.9. Bar,  $5 \mu\text{M}$ .

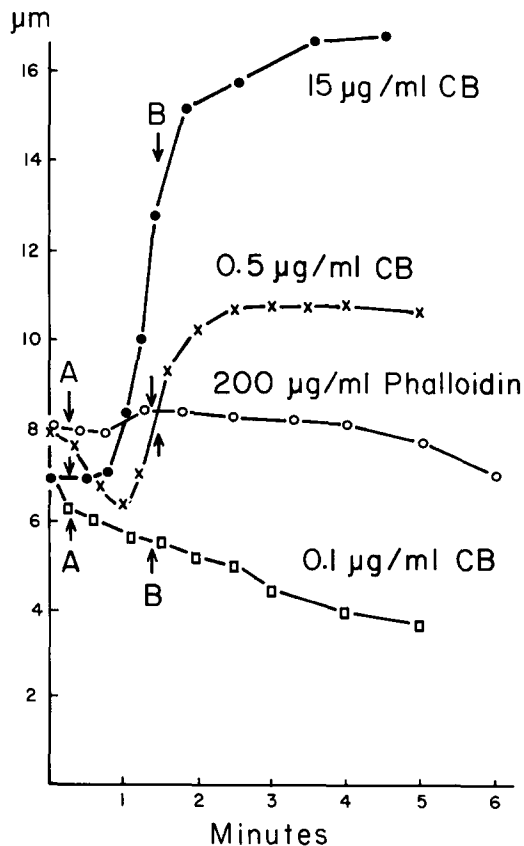


FIGURE 11 Change in furrow diameter after lysis in cytochalasin B (CB) or phalloidin. In the phalloidin experiment, cleavage was delayed for 1.5 min by elevation of the free calcium concentration in solution A only. In the cytochalasin B (CB) experiments, 1% dimethyl sulfoxide was also present in the lysis medium.

occurs in cells that have just begun to furrow. This suggests that a threshold of contractile ring organization must be reached in the unlysed cell for cleavage to continue after lysis. The largest isotropic contractions are observed in telophase cells that have not yet established a furrow and are incapable of cleavage after lysis. This result is consistent with theoretical models of furrow initiation that suggest that recruitment of mechanochemical elements from the rest of the cortex into the furrow is involved in establishing the contractile ring (21, 29, 33).

The findings presented here are in agreement with those reported by Hoffman-Berling in 1954 (15, 16). The rates of cleavage after lysis in Brij 58-polyethylene glycol are faster than that described in glycerinated fibroblasts, but the extent of cleavage after lysis is similar in the two systems. In both cell model systems, after lysis, optimal cleavage requires ATP, has a pH optimum around neutrality, and is inhibited by high ATP concentrations. Because Hoffman-Berling used phosphate buffers, he was unable to describe calcium ion requirements for cleavage. He also found that inhibitors of actomyosin-based contractions, such as sulfhydryl reagents, inhibited cleavage in the glycerinated cell model.

#### Cleavage Inhibition

Cleavage does not occur after lysis in the presence of the fungal metabolites phalloidin and cytochalasin B, the protein NEM-S<sub>1</sub>, or in suboptimal physiological conditions. With the exception of phalloidin, these treatments lead not only to a blockage of furrow constriction but also to an increase in furrow diameter. In addition, the isotropic cell contractions are blocked and the dimensions of the permeabilized cell model may increase slightly. Furrow relaxation was dramatic and occurred rapidly in the presence of high calcium, alkaline pH,

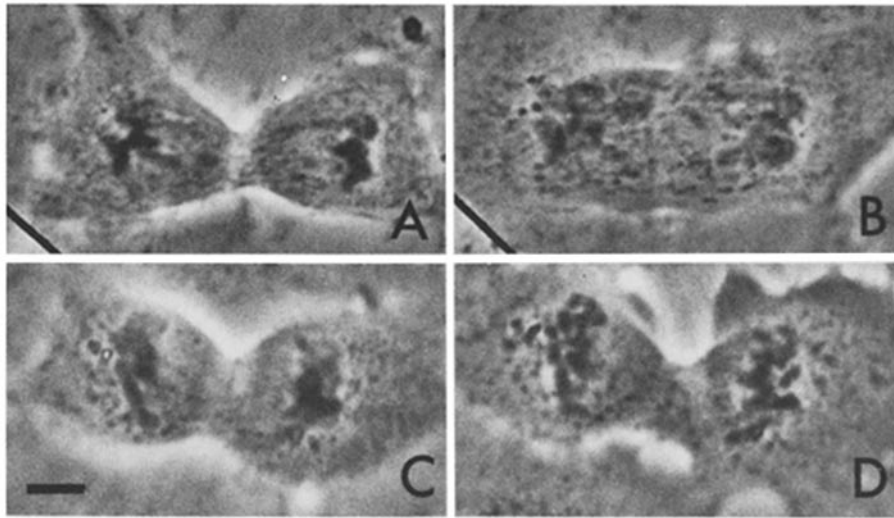


FIGURE 12 Cleavage after lysis in cytochalasin B or phalloidin. A and C are phase micrographs of cells just before addition of lysis medium. B is the corresponding cell 2 min after lysis in 15  $\mu\text{g}/\text{ml}$  cytochalasin B and D is the corresponding cell 5 min after lysis in 200  $\mu\text{g}/\text{ml}$  phalloidin. Bar, 5  $\mu\text{m}$ .

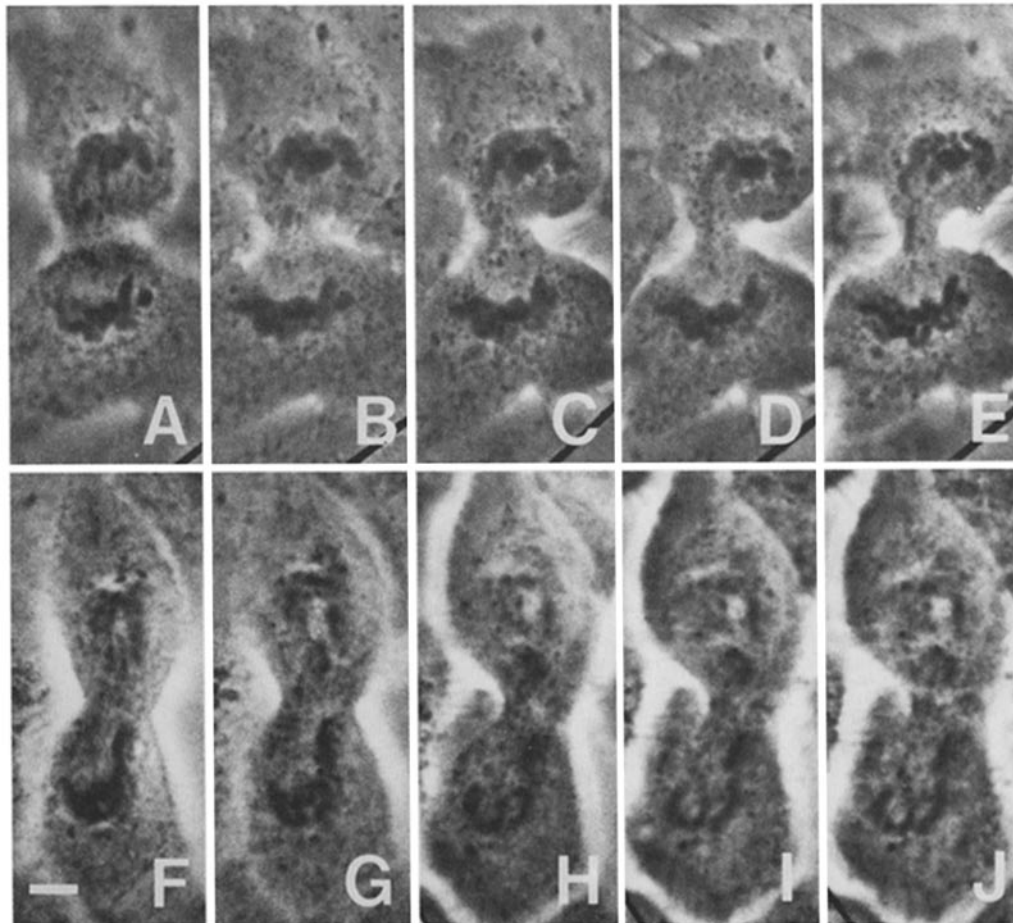


FIGURE 13 Selected frames from a ciné record of cells lysed in  $S_1$  or NEM- $S_1$ . In A-E, a cell was lysed in 1.9 mg/ml  $S_1$ , in F-J, a cell was lysed in 1.6 mg/ml NEM- $S_1$ . Because solution A contained 4  $\mu\text{M}$  free calcium and no ATP, cleavage was delayed for 1.5 min after lysis until solution B was added. A and F were taken before lysis; B and G, 1 min after lysis; C and H, 2 min after lysis; D and I, 4 min after lysis; and E and J, 6 min after lysis. Bar, 5  $\mu\text{m}$ .

and cytochalasin B, but in 10 mM ATP and in NEM- $S_1$ , furrow diameter increased slowly and relaxation often did not occur until several minutes after lysis. These observations suggest that cytochalasin B, alkaline pH, and high calcium act directly on the contractile ring to disorganize and relax the furrow, and

that NEM- $S_1$  and high ATP have an indirect effect on furrow integrity.

Although there is ample evidence that cytochalasin B interferes with the function of many microfilament systems, including those involved in cleavage, the mechanism of cytochalasin



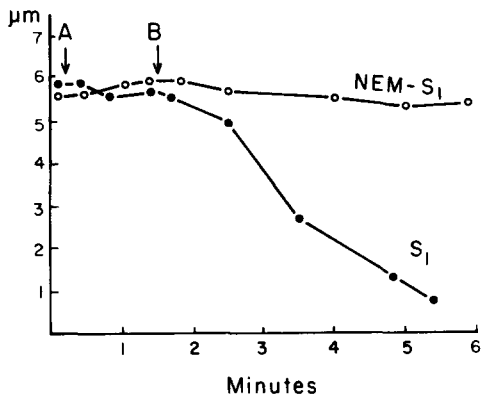


FIGURE 14 Change in furrow diameter after lysis in NEM-S<sub>1</sub> or S<sub>1</sub>. Cells were lysed in 2.0 mg/ml NEM-S<sub>1</sub> or S<sub>1</sub>. Cleavage was delayed for 1.5 min after lysis by raising the free calcium level in solution A.

B action is still controversial (see reference 38 for review). At high concentrations (10–20 μg/ml), cytochalasin B solates actin gels; at lower concentrations the drug may block actin subunit addition to the growing end of the microfilament (12, 13). It has also been suggested that cytochalasin B disrupts microfilament-membrane attachments (38). In unlysed PtK<sub>1</sub> cells, cytochalasin B promotes a rapid dissolution of the furrow and relaxation of the cytoplasm to restore the original early telophase cell profile. Cytochalasin B addition at lysis causes an immediate collapse of the furrow and a severalfold increase in furrow width. Cytochalasin B also blocks the isotropic shape changes observed after lysis. These results are consistent with a role of cytochalasin B in directly promoting disassembly of the mechanical elements of the furrow and the surrounding cortex, perhaps by dissolving actin filaments or by disorganizing the actin gel.

The increase in furrow width in 10<sup>-5</sup> M Ca<sup>++</sup> and alkaline pH may also be caused by a promotion of contractile ring disassembly. In a recent review, Taylor and Condeelis (39) have argued that solation of actin gels is a necessary and regulatory step during the contractile cycle required for amoeboid movement. In normal cleavage, contractile ring disassembly and contraction may be closely coupled, but after cytochalasin B treatment, high pH and high calcium disassembly may occur more rapidly than contraction. This would result in furrow relaxation. There have been several reports that calcium and pH can regulate the extent of gelation of cytoplasmic extracts (for review see references 14 and 39). For example, Mimura and Asano (25) have purified two protein factors from Ehrlich ascites cells that promote actin gelation in submicromolar free calcium and solation in micromolar free calcium ions. Calcium or pH may play a similar role in regulating the disassembly of microfilaments in the contractile ring as cleavage progresses.

NEM-S<sub>1</sub> and 10 mM MgATP may inhibit cleavage by interfering with actomyosin interactions in the contractile ring. We have previously reported that NEM-HMM forms rigorlike bonds even in the presence of ATP and blocks actomyosin interactions in several model systems by preventing unmodified myosin from interacting with the actin microfilament (24). NEM-S<sub>1</sub> probably inhibits cleavage by a similar mechanism, that is, it may prevent native myosin from interacting with filaments in the contractile ring. High ATP has been demonstrated to plasticize glycerinated myofibrils and glycerinated dividing cells (15, 16, 37). Szent-Györgyi (37) has suggested that under these circumstances myosin is cleaving ATP without

interacting with actin and generating tension. This interpretation of the mode of action of high ATP in blocking furrow contraction is supported by the nucleotide specificity of the relaxation, which is similar to the nucleotide specificity of myosin (26). Cleavage in the presence of 10 mM CTP and GTP may be caused by the presence of endogenous transphosphorylases or endogenous ATP in the cell model. The gradual relaxation of the furrow after lysis in NEM-S<sub>1</sub> and high ATP suggests that actomyosin interactions, i.e., rigor bonds, contribute to the integrity of the furrow and may be required to help maintain the contractile ring structure. A similar phenomenon has been described by Crooks and Cooke (6) who found that high ATP broke actomyosin threads.

An alternative interpretation of these results is that NEM-S<sub>1</sub> and high ATP may be selectively solubilizing components necessary for successful cleavage. For example, NEM-S<sub>1</sub> may displace cross-linking proteins that bind to actin and are required to hold the contractile ring together. High ATP may be capable of selectively solubilizing myosin.

Phalloidin is a drug known to stabilize microfilaments in vitro and block actin depolymerization without interfering with actomyosin interactions (7). Cleavage in permeabilized cells is blocked by concentrations that block shuttle streaming in *Physarum* and locomotion in microinjected fibroblasts (36, 42). In the lysis experiments, it is not clear whether phalloidin interferes with cleavage by inhibiting essential actin depolymerization within the furrow region or by changing the mechanical properties of the cortex outside the furrow by promoting microfilament formation throughout the cell. In either case, phalloidin inhibition of cleavage is consistent with the morphological observations of the transient nature of the contractile ring (31).

### Calcium and Cleavage

It has been estimated that the free calcium level in resting cells, such as unstimulated nerve and muscle, is between 0.05 and 0.1 μM (for review see reference 19). Because cleavage continues after lysis at these levels, a large increase in intracellular calcium is not required for maintenance of furrowing activity. This result is unlike smooth or striated muscle in which the contractile machinery is insensitive to 0.1 μM free calcium and maximum tension is generated in the presence of 1 μM calcium (8). Because the extent of cleavage after lysis is reduced by addition of EGTA to the medium, there must be a minimum calcium requirement for cleavage. There is also a maximal calcium concentration which is unfavorable for continued constriction and which leads to furrow relaxation rather than constriction. Preliminary experiments with a more precisely defined Ca/EGTA buffer suggest that the optimal range of free calcium that supports cleavage at pH 6.94 is between 0.1 μM and 0.6 μM. This calcium level is almost identical to that described by Hellewell and Taylor (14) for solation of actin gels prepared from *Dictyostelium* amoebae.

Baker and Warner (4) have demonstrated that amphibian eggs microinjected with EGTA do not undergo cleavage. Furrowlike contractions have also been elicited in eggs with calcium ionophores and calcium-enriched medium (1, 34). Because the calcium levels required for constriction in lysed cells are so low, these experiments on eggs may define a requirement for calcium for furrow activation and assembly rather than furrow function. By analogy with smooth muscle (35), this initiation event could be a calcium-stimulated phosphorylation of myosin light chains that activates the myosin involved in

cleavage. After cleavage is initiated, lower levels of calcium would be permissible for furrow constriction and could coordinate contractile ring disassembly. Taylor and Condeelis (39) have argued that local breakdown of actin gels is required for contraction during amoeboid movement. Although the organization of the contractile ring is very different from that of the amoeba cytoplasm, a similar process of controlled dissolution of structure coupled to contraction may be required for successful cleavage.

With the permeabilized cell model, it will now be possible to evaluate the role of phosphorylation and other possible regulatory mechanisms during cleavage. Experiments are in progress to define the role of calcium during furrow initiation and contraction and to examine the ultrastructure of the cleavage furrow in the permeabilized cell.

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