

On the Mechanism of Anaphase A: Evidence that ATP Is Needed for Microtubule Disassembly and Not Generation of Polewards Force

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Abstract. As anaphase began, mitotic PtK₁ and newt lung epithelial cells were permeabilized with digitonin in permeabilization medium (PM). Permeabilization stopped cytoplasmic activity, chromosome movement, and cytokinesis within about 3 min, presumably due to the loss of endogenous ATP. ATP, GTP, or ATP- γ -S added in the PM 4–7 min later restarted anaphase A while kinetochore fibers shortened. AMPPNP could not restart anaphase A; ATP was ineffective if the spindle was stabilized in PM + DMSO. Cells permeabilized in PM + taxol varied in their response to ATP depending on the stage of anaphase reached: one mid-anaphase cell showed initial movement of chromosomes back to the metaphase plate upon permeabilization but later, anaphase A resumed when ATP was added. Anaphase A was also reactivated by cold PM ($\sim 16^{\circ}\text{C}$) or PM containing calcium (1–10 mM). Staining of fixed cells with antitubulin showed that microtu-

bules (MTs) were relatively stable after permeabilization and MT assembly was usually promoted in asters. Astral and kinetochore MTs were sensitive to MT disassembly conditions, and shortening of kinetochore MTs always accompanied reactivation of anaphase A. Interphase and interzonal spindle MTs were relatively stable to cold and calcium until extraction of cells was promoted by longer periods in the PM, or by higher concentrations of detergent.

Since we cannot envisage how both cold treatment or relatively high calcium levels can reactivate spindle motility in quiescent, permeabilized, and presumably energy-depleted cells, we conclude that anaphase A is powered by energy stored in the spindle. The nucleotide triphosphates effective in reactivating anaphase A could be necessary for the kinetochore MT disassembly without which anaphase movement cannot proceed.

No consensus currently exists regarding the role of ATP during anaphase A (chromosome-to-pole movement) and it is difficult to evaluate the diverse data on this subject. We (Pickett-Heaps and Spurck, 1982; Spurck et al., 1986a, b) have found that the concentration of metabolic inhibitors needed to cause rapid (within 1 min) and completely reversible cessation of cellular activity (presumably by depleting cellular ATP levels) in live cells is critical; 2,4 dinitrophenol (DNP)¹ is effective in both diatoms and mammalian cells only at the seemingly high concentration of 1 mM, and lowering the concentration even to 0.5 mM results in a significantly delayed and often incomplete response. In contrast, far lower concentrations of metabolic inhibitors were used in most previous work (reviewed by Spurck et al., 1986a, b; Hepler and Palevitz, 1986). Furthermore, many who work with animal cell systems use inhibitors of oxidative phosphorylation alone. We (Spurck et al., 1986a) found that glycolysis must also be blocked by using

2-deoxyglucose (DOG) in conjunction with DNP or other metabolic inhibitors. Without DOG, cellular activity may be maintained in DNP, and glycolysis, we believe, keeps cells alive for the long periods of DNP treatment alone (1–4 h or more) reported in some papers (e.g., De Brabander et al., 1982, and others). For such reasons, it is difficult to assess earlier work on the effect of ATP depletion on live cells when glycolysis could have continued. Mazia's review (1961) summarizes evidence that cells have an energy reservoir that permits mitosis to proceed in metabolic inhibitors. Thus, the effects reported in this work may have been due to long-term poisoning in low concentrations of single metabolic inhibitors.

Recently, we (Spurck et al., 1986a) and Hepler and Palevitz (1986) have found that anaphase in PtK cells and *Tradescantia* stamen hairs is reversibly blocked by metabolic inhibitors. We concluded that the ATP required during anaphase A might be for the disassembly of kinetochore microtubules (MTs) without which anaphase A cannot proceed, rather than for activating a motility system involved in generating polewards motion. If so, then in animal cells as well as diatoms (Pickett-Heaps et al., 1984b), anaphase A could be

1. *Abbreviations used in this paper:* AMPPNP, adenylyl-imidodiphosphate; ATP- γ -S, adenosine-5'-O-[3-thiotriphosphate]; DNP, 2,4 dinitrophenol; DOG, 2-deoxyglucose; GTP- γ -S, guanosine-5'-O-[3-thiotriphosphate]; MT, microtubule; PM, permeabilization medium.

powered by the release of energy stored in the spindle. This paper addresses that issue directly.

To investigate these matters further, we have had to use a permeabilized cell model based on those developed by others (see Discussion). This necessity arose from the fact that energy-depleted cells cannot survive the trauma of microinjection (Spurck et al., 1986a). Thus we could not use this technique to test whether various substrates (e.g., ATP) unable to cross the cell membrane, could reverse the effects generated by DNP/DOG. We demonstrate that in permeabilized PtK and newt lung cells, anaphase A can be reactivated even in the continued presence of DNP/DOG by several high energy compounds. Moreover, reactivation can progress in the absence of ATP under conditions that promote disassembly of kinetochore MTs.

Materials and Methods

Cell Culture

PtK₁ cells were grown in Corning 75-cm² tissue culture flasks in Ham's F-12 medium (Gibco, Grand Island, NY) supplemented with 10% FCS and 0.03% ampicillin. They were subcultured onto Chromerge-cleaned coverslips 48 h before use. Newts (*Taricha granulosa*) collected in Oregon were kept at room temperature in deionized water and fed live brine shrimp weekly. Newt lung explants were placed on carbon-coated coverslips in a drop of filter-sterilized medium containing 60% L-15 (KC Biological Inc., Lenexa, KS), 10% FCS (Flow Laboratories Inc., McLean, VA), 0.106% BES (*N,N*-bis[2-hydroxyethyl]-2 aminoethane sulphonic acid), 0.151% Pipes (Sigma Chemical Co., St. Louis, MO), and 5% whole egg ultrafiltrate (Gibco; a gift from Dr. C. Rieder) in deionized water (pH at 7.5). The explants were maintained in a 26°C incubator with fresh newt medium added on the third day. Cell divisions typically occurred 10–12 d after the initial cell plating.

Cell Permeabilization

Cells cultured on coverslips were mounted in perfusion chambers (Pickett-Heaps and Spurck, 1982) which allow for the rapid replacement of culture medium with any chosen medium via a two-way stopcock, while the cells are continuously monitored. The cells were permeabilized at the onset of anaphase A or anaphase B by admission of the permeabilization medium (PM). This PM was based on a lysis buffer (Rozdzial and Haimo, 1986) which contains 30 mM Hepes, 0.5 mM EDTA, 5.0 mM MgSO₄, 10 mM EGTA, 33 mM K-acetate, and 2.5% polyethylene glycol (mol wt 20,000); the PM was prepared bimonthly and stored at 4°C. Digitonin (from Sigma; made fresh as a 0.1% stock solution in 1:1 water/ethanol) was diluted into PM to make a final concentration of 0.0005%. A blend of protease inhibitors was added fresh to the PM before the experiments; the blend included Aprotinin at 1.0 µg/ml (Sigma Chemical Co.), Leupeptin at 1.0 µg/ml (stock solution: 5 mg/ml in DMSO kept at -20°C), DL-DTT at 1.5 µg/ml (from Sigma Chemical Co.; stock solution 15 mg/ml in H₂O kept at -20°C), both aliquoted in amounts to avoid refreezing; the EDTA in the PM also acts as a protease inhibitor.

The nucleotides ATP and GTP (1–10 mM), along with the hydrolyzable analogs ATP-γ-S (adenosine-5'-O-[3-thiotriphosphate]; 0.1 mM), GTP-γ-S (guanosine-5'-O-[3-thiotriphosphate]; 0.1 mM) and the ATP competitive inhibitor AMPPNP (adenyl-imidodiphosphate; 0.1 mM), were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN, and were added to the PM before use (pH of the PM was 7.4); they were admitted to the chamber via the two-way stopcock as required. In some experiments, the metabolic inhibitors DNP and DOG, each at 1 mM (Spurck et al., 1986a), were used to stop anaphase before permeabilization (see Results). In other experiments, calcium chloride (1–10 mM) from Fisher Scientific Co. (Pittsburgh, PA) was dissolved directly in PM and passed into the chamber after permeabilization.

To test the extent of permeabilization, the DNA-binding fluorochrome Bisbenzimidazole (Hoechst No. 33258; mol wt 562, from Sigma Chemical Co.) (4 mg/ml) was diluted directly into the perfusion medium (see Results). Light and immunofluorescence microscopy were carried out as previously described (Spurck et al., 1986a, b) using an antitubulin kindly supplied by Dr. D. Asai (Asai et al., 1982).

Cold Treatments

Cells which had been permeabilized at 36°C were perfused with cooled PM by running the inlet tube connecting the stopcock and perfusion chamber (see above) through an ice bath. In addition, the perfusion chamber was isolated from the warmed microscope stage via strips of teflon. Cold PM then flowed continuously through the chamber for the duration of the experiment. A thermistor probe inserted into the efflux tube monitored the temperature (15–17°C) of the PM as it left the chamber. In control experiments, live cells were similarly chilled and then rewarmed to confirm that this cooling procedure reversibly breaks down the spindle as expected. In other experiments, DMSO (Fisher Scientific Co.) was added at 0.15 M to the cold PM during and after permeabilization.

Microinjection

PtK₁ cells were microinjected with the nontoxic fluorochrome, 5(6)-carboxyfluorescein (from Eastman Kodak Co., Rochester, NY) diluted in injection buffer, using the protocol previously described (Spurck et al., 1986a). After the cells were allowed to recover from the injection trauma (10–15 min), they were permeabilized with various concentrations of digitonin in PM and observed with a Zeiss IM-35 inverted microscope using epifluorescence.

Results

We have been unable to restart anaphase A consistently using published protocols for permeabilizing cells (e.g., Stearns and Ochs, 1982; Clarke and Rosenbaum, 1984; Cande, 1982b; Sakai et al., 1979; Snyder, 1981) and cannot account for this problem. With the advice of Dr. M. Rozdzial, we modified his permeabilization medium (PM; Rozdzial and Haimo, 1986) which contains the cholesterol-intercalating detergent digitonin (see Materials and Methods). This PM was reliable and many reactivated spindles were followed photographically. We estimate our success rate as ~90% in reactivating some mitotic activity after cessation of all movement. For obtaining measurable reactivation of anaphase A, the timing of permeabilization is critical (see below).

Extent of Permeabilization

Cells exposed to a 4 mg/ml solution of the DNA-binding fluorochrome Hoechst 33258 in the PM were subsequently observed under epifluorescence for staining of their chromatin or chromosomes. This fluorochrome was unable to cross the cell membrane of control cells incubated in either culture medium or in PM without digitonin. However, cells in PM containing 0.0005% digitonin and the Hoechst stain exhibited DNA fluorescence after 2 min, with fluorescence increasing thereafter. This result indicates that small molecules can diffuse across the cell membrane under these permeabilization conditions. Second, live cells were microinjected with the fluorescent dye 5(6)-carboxyfluorescein (mol wt 376). After ~10 min, no leakage of this stain was observed either from microinjected control cells or from cells exposed to PM without digitonin; slow fluorescence leakage was observed after 10 min from cells in PM containing 0.0005% digitonin. Increasing the concentration of digitonin 10-fold resulted in a rapid loss of the fluorescent stain.

Two observations are pertinent to the reliability of this permeabilization protocol. First, on only a few occasions (9 out of 135 experiments), cells continued through anaphase for ~5–10 min after the PM was admitted to the chamber. We suspect that these cells were not permeabilized quickly enough to prevent anaphase from continuing (the reason we always made sure anaphase had stopped before attempting

reactivation). Second, when examining control cells permeabilized for 10 min in PM containing Ca^{++} (see below) and then fixed and stained with antitubulin, microtubules (MTs) had virtually disappeared in a few interphase cells, in contrast to the large number of less affected adjacent cells. We presume that these cells had, for unknown reasons, suffered more extraction than usual since increasing cytoplasmic extraction coincides with markedly lessened stability of MT systems in permeabilized cells. If so, cells may be variable in their response to permeabilization.

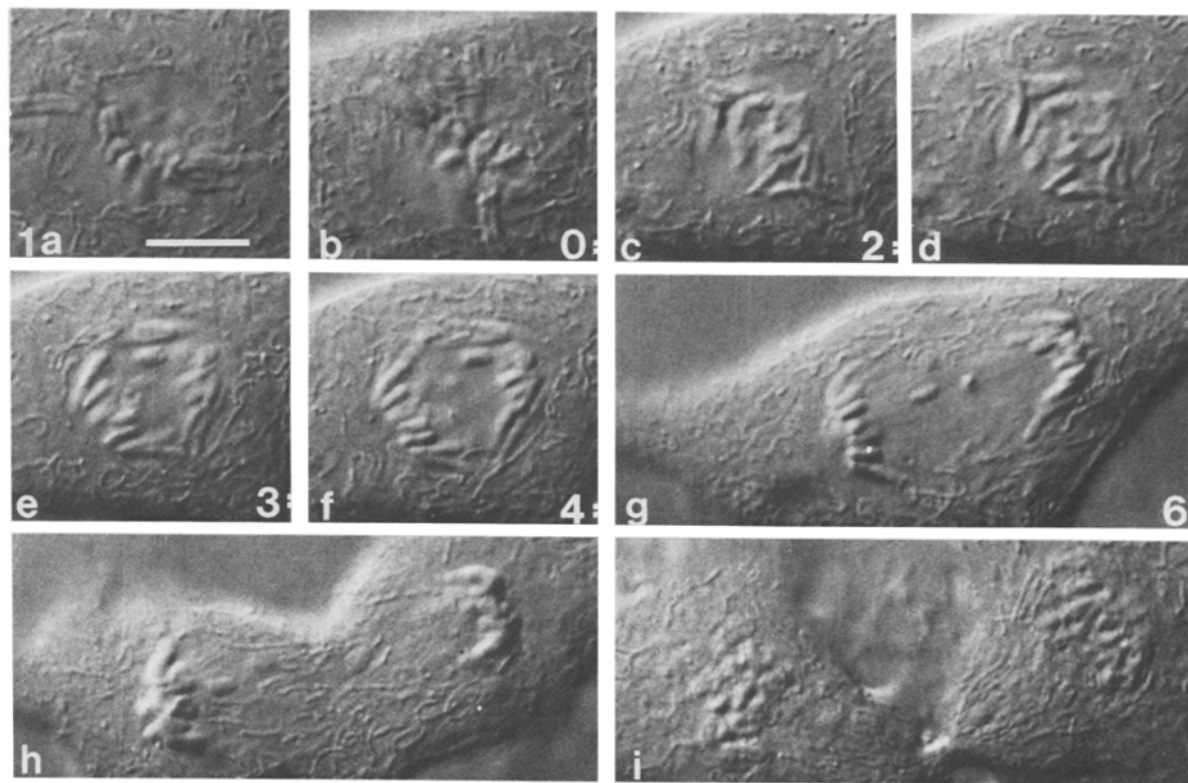
Spindle Behavior after Permeabilization

The course of normal division is shown in Fig. 1; most of anaphase A is accomplished in $\sim 5\text{--}7$ min (Fig. 1, *b-f*) and reaches completion (Fig. 1, *g* and *h*) as anaphase B becomes pronounced. Treatment of cells with PM containing 0.0005% digitonin caused poleward movement of chromosomes to cease about 2–3 min after opening the stopcock to the experimental chamber if the permeabilization was initiated at the onset of anaphase. This delay can be attributed to the time taken to pass the PM into the chamber, permeabilize the cell, and allow the endogenous ATP (and other small molecules) to diffuse out. Cells permeabilized before sister chromatid separation were unable to enter anaphase; cells permeabilized later than 1 min after chromatid separation usually continued anaphase A for a distance sufficient to make the subsequent reactivation difficult to measure. If cells were permeabilized at the moment of sister chromatid separation,

the chromosomes stopped moving well before reaching the pole and reactivated anaphase A movement was therefore easily measurable. Fig. 2 shows a cell permeabilized a little later than in normal experiments so that chromosome separation can be seen to have occurred. Typically (e.g., Figs. 3 *c*, 5 *d*, 6 *e*, 8 *b*), the chromosomes became briefly less distinct at permeabilization (Fig. 2 *c*), a useful indicator that permeabilization had indeed occurred. There was no significant anaphase A movement in cells kept in PM alone after the first couple of minutes. The spindle region (clear area of cytoplasm) steadily lost its outward form (Fig. 2, *d-f*, and Fig. 8, *a* and *b*) in permeabilized cells, but this invasion of the spindle by cytoplasmic granules was not accompanied by any obvious alterations in spindle MTs visualized by immunofluorescence staining of fixed cells (Fig. 12, *b* and *c*) or observations with polarizing optics. Thus, the position of the poles could not be determined confidently after permeabilization and so we measured the extent of reactivated chromosome movement from the separation achieved by kinetochores.

Reactivation of Anaphase A by Nucleotides

PtK₁ and newt cells permeabilized at the onset of anaphase were left in the PM for 4–7 min to ensure adequate permeabilization. By this stage, chromosomes had stopped moving, thus confirming that permeabilization had occurred and that any subsequent reactivation was not due to continuation of anaphase in a poorly permeabilized cell (see above). Next, either 1 mM ATP/Mg⁺⁺ or 1 mM GTP/Mg⁺⁺ was added in



On figures, PM denotes permeabilization, which is always at zero time ($T = 0$ min). Other times measured from this moment. The treatments given are also shown on the figures.

Figure 1. Control PtK cell; normal mitosis and cytokinesis. (*a*) Metaphase. (*b*) Initiation of anaphase. Typically, this is $T = 0$ min when permeabilization would be initiated. (*c* and *d*) Mid-anaphase ($T = 2$ min). (*e* and *f*) Later anaphase A ($T = 3$ and 4 min, respectively). (*g*) Anaphase B in progress. (*h* and *i*) Cleavage. Bar, 10 μM .

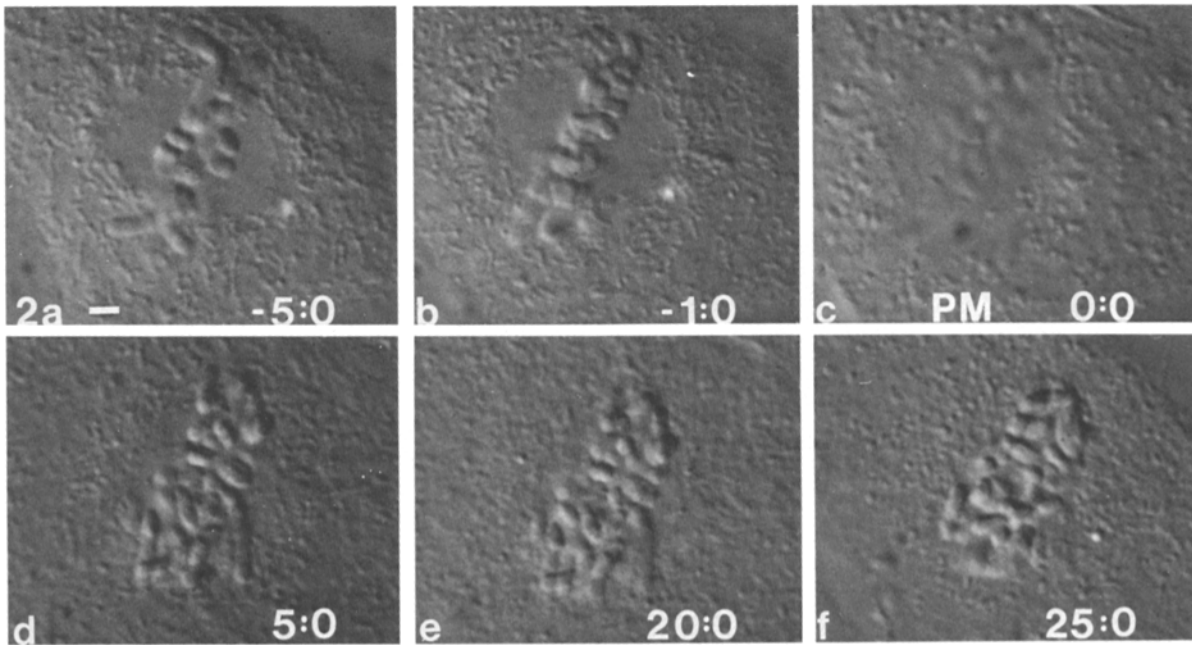


Figure 2. Control PtK cell permeabilized (PM) at T = 0 min (c) and not reactivated. (a) Metaphase, 5 min before permeabilization. (b) Initiation of anaphase. (c) Permeabilization, delayed slightly to ensure visible chromatid separation in control. (d-f) Spindle in PM at T = 5, 20, and 25 min, respectively. Bar, 1 μ m.

the PM; in most cells, the arrested chromosomes resumed poleward movement (bottom cell in Fig. 3). The extent to which anaphase B may have contributed to the separation of chromosomes was difficult to assess. Cells rarely showed significant spindle elongation when permeabilization was initiated at the beginning of anaphase; anaphase A was clearly evident by the chromosomes becoming clustered towards the poles. A common complication was the tendency of dividing cells to round up upon permeabilization (as often happens anyway during normal mitosis); reactivation still occurred but it was difficult to record.

Reactivation of Anaphase A in Energy-depleted Cells

In another approach, we induced ATP depletion in live cells using DNP/DOG (1 mM each for 5-7 min; Spurck et al., 1986a, b). After cessation of anaphase and all cytoplasmic movement, the cells were permeabilized with PM containing DNP/DOG. When these cells were subsequently treated with ATP (1 mM) in PM + DNP/DOG, we observed reactivated anaphase A movement (Fig. 4). Sometimes we added the DNP/DOG at the moment of permeabilization (Fig. 5 c) to stop any endogenous production of ATP (e.g., by glycolysis and still-functioning mitochondria). In practice, this

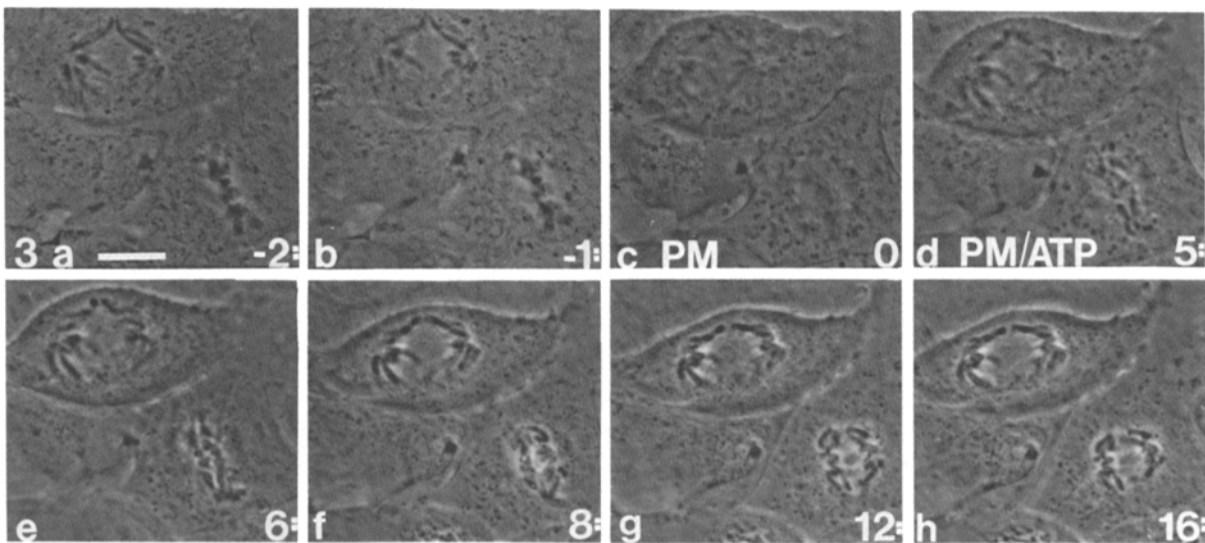


Figure 3. Two spindles undergoing anaphase A and B. (a) 2 min before permeabilization. (b) Initiation of anaphase A in lower cell. (c) Permeabilization (PM); T = 0 min. (d) ATP added at T = 5 min. (e-h) Progression of anaphase A in lower cell; some anaphase B in upper cell as well. Bar, 10 μ m.

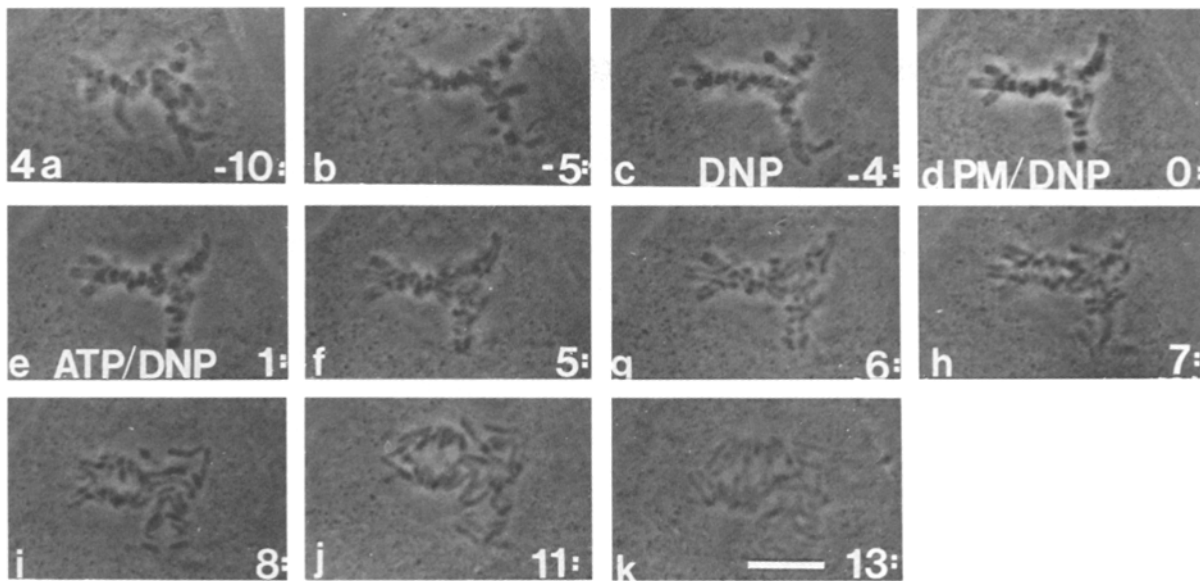


Figure 4. Reactivation of energy-depleted, tripolar PtK cell. (a and b) Metaphase. (c) Treatment with DNP/DOG at initiation of anaphase, 4 min before permeabilization. All cytoplasmic activity stopped within 1–2 min. (d) Permeabilization in PM + DNP/DOG (T = 0 min). (e–k) Reactivation of anaphase A by addition of ATP to the PM + DNP/DOG. Bar, 10 μ M.

seemed to have little effect in hastening cessation of chromosome movement, and normal reactivation of anaphase A was recorded with ATP in the PM + DNP/DOG (Fig. 5).

Reactivation by Analogs

PM containing 0.1 mM of the analog ATP- γ -S induced reactivation of chromosome movement (Fig. 6); the few times we tried it, GTP- γ -S (0.1 mM) worked equally well. In contrast, permeabilized cells treated with the nonhydrolyzable analog AMPPNP (0.1 mM) did not show any anaphase A movement, nor did reactivation occur when this reagent was subsequently replaced by 1 mM ATP.

Effects of Ca^{++} and Cold on Permeabilized Cells

We directly tested whether the ATP that reactivated anaphase A is functioning through a spindle motor, or whether it might be involved in MT disassembly (Spurck et al., 1986b). Spindle MTs are sensitive to Ca^{++} (Salmon, 1982; Weisenberg and Deery, 1981; Kiehart, 1981); accordingly, after anaphase movement had been halted for 4–7 min with PM, 1 mM Ca^{++} was washed in with additional PM. This addition of Ca^{++} resulted in a resumption of chromosome motion to the poles (Fig. 7). The level of free Ca^{++} under these conditions is difficult to estimate but must be very low ($\sim 10^{-8}$ M; Porrello and Burnside, 1984); increasing the amount of $CaCl_2$

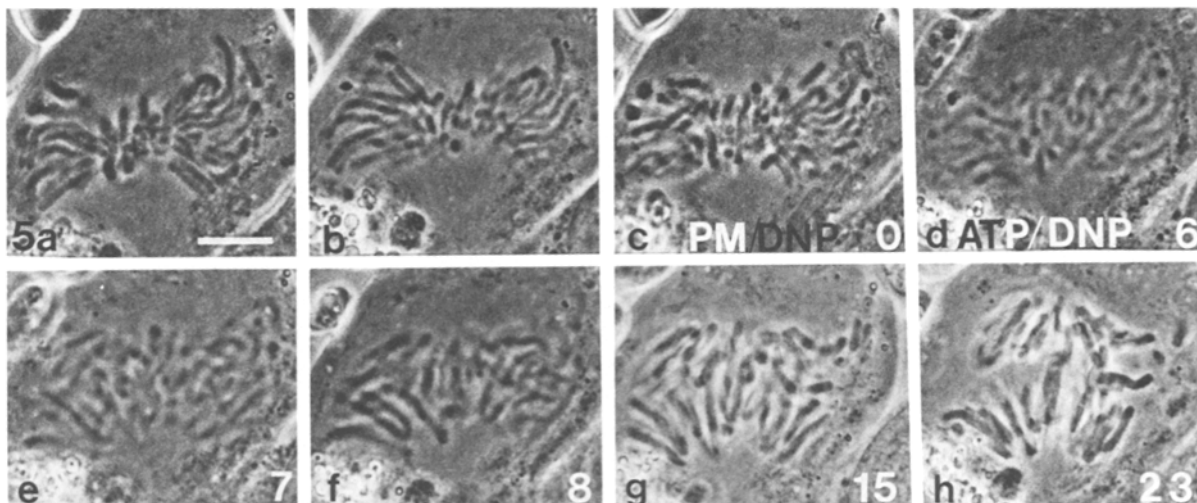


Figure 5. Similar to cell in Fig. 4, using newt lung cell. (a and b) Metaphase and initiation of anaphase. (c) Permeabilization in PM + DNP/DOG; chromatids well separated, but quickly stopped. (d–h) Reactivation of anaphase A by adding ATP to the PM + DNP/DOG. Bar, 10 μ M.

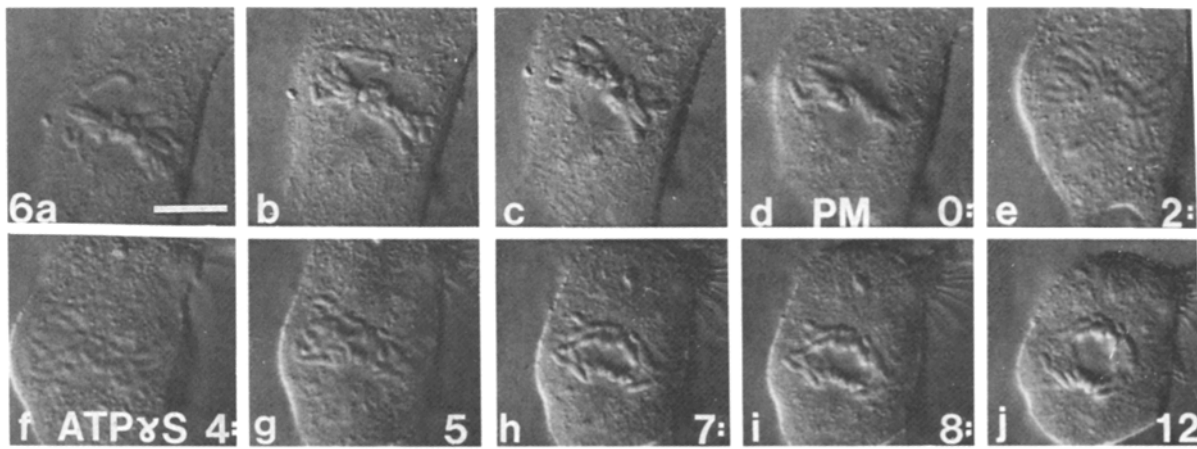


Figure 6. PtK cell; anaphase A reactivated with ATP- γ -S. (a-c) Metaphase. (d) Permeabilization at initiation of anaphase A. (e) Some chromosome separation before anaphase A stopped. (f) Addition of ATP- γ -S at T = 4 min. (g-j) Reactivation of anaphase A, ceasing after \sim 12 min. Bar, 10 μ M.

in the PM to 5–10 mM ($\sim 5 \times 10^{-7}$ M) gave a quicker and apparently (i.e., subjectively) more definitive response.

Cold treatment can also disassemble spindle MTs (Fuseler, 1975). If permeabilized anaphase cells were perfused with cool ($\sim 16^\circ\text{C}$) PM, again, anaphase A resumed but at a slower rate (Table I) than ATP or Ca^{++} reactivation (Fig. 8). These experiments were carried out using only the PtK₁ cells (newt spindles are more resistant to cold since newts are poikilotherms). While some anaphase A was almost always observed with the chromosomes clustered at the poles, the extent of chromosome movement was often less than normal and less easy to measure. In addition, cold and some calcium-treated spindles were noticeably shorter (i.e., under MT disassembly conditions), suggesting that anaphase B

was not contributing to chromosome movement in these experiments. However, other Ca^{++} -treated spindles did show slight elongation.

Three types of controls were run in order to check the stability of MTs under the experimental conditions used. First, live metaphase cells, observed with polarization optics, were subjected to cold PtK medium the same way as the permeabilized cells. The spindles disassembled in ~ 10 min (Fig. 9), distinctly slower than in permeabilized cells; these mitotic cells reformed spindles within 12 min and proceeded with mitosis when the cool culture medium was warmed up to 37°C . Secondly, permeabilized metaphase cells were similarly followed; their spindles appeared quite stable in PM alone (i.e., over about 15 min, as in Fig. 10, a and b) but

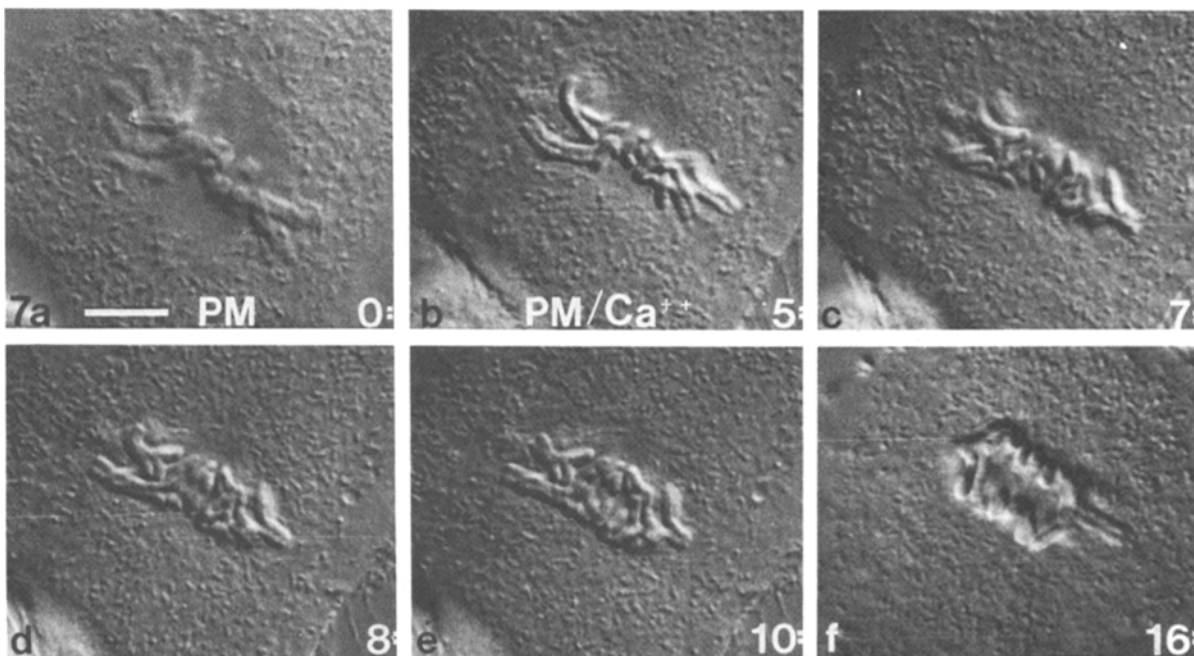


Figure 7. PtK cell reactivated with 1 mM Ca^{++} in PM. (a) Permeabilized following this frame at very early anaphase. (b) PM + Ca^{++} added, T = 5 min. (d-f) Reactivation of anaphase A. Bar, 10 μ M.

Table I. Effect of Treatments on Anaphase A

Treatment	Cells measured	Extent of K/K separation	Rate
	<i>n</i>	μm	$\mu\text{m}/\text{min}$
Average metaphase K/K distance	10	4.0 ± 1.3	—
Control	10	11.9 ± 1.3	1.2 ± 0.2
ATP (1.0 mM)	6	9.8 ± 1.8	1.0 ± 0.2
ATP/DNP (0.1 mM/1.0 mM)	4	9.1 ± 4.0	0.6 ± 0.2
Calcium (1.0 mM)	8	10 ± 1.8	0.9 ± 0.2
ATPyS (0.1 mM)	4	9.6 ± 1.7	0.9 ± 0.4
16°C	8	8.4 ± 2.1	0.7 ± 0.1
16°C + DMSO (150 mM)	4	4.3 ± 0.9	0.1 ± 0.1
PM w/0.0005% digitonin	4	4.0 ± 1.2	0.1 ± 0.1
AMPPNP (0.1 mM)	3	4.4 ± 0.4	0

Summary of approximate rates of anaphase A measured from batches of cells undergoing the different experimental treatments 4–5 min after permeabilization. Measurements taken of kinetochore separation (K/K), which typically is 4 μm (top line) at metaphase.

when treated with cold PM, began to shrink within 1.5 min, and were undetectable by 6.5 min. The same result was observed when similar spindles were exposed to 1 mM Ca^{++} in PM (Fig. 10). Thirdly, the response of some cold-treated cells was recorded photographically and then the cells were fixed with cold methanol and their position marked on the coverslip with a diamond scribe; they were subsequently stained with antitubulin. These fixations were not as good as those obtained with similar coverslips treated directly with methanol at -20°C (see below); however, very short dense kinetochore and interzonal fibers were obvious in these and other nearby anaphase cells (not shown). These results all

confirm that the treatments we used did disassemble the spindle.

Effects of Microtubule-stabilizing Agents on Reactivation

DMSO acts as an MT-stabilizing agent (Magistrini and Szollosi, 1980). When experiments were repeated using cold PM (16°C) containing 0.15 M DMSO, anaphase A could not be reactivated (Fig. 11); when the DMSO was removed, continued cold treatment did not reactivate chromosome movement.

Another well-characterized MT-stabilizing agent is taxol. With limited quantities (a gift from Dr. Jon Scholey), we could only perform three runs on permeabilized cells. Addition of PM + taxol caused anaphase to stop, and in a mid-anaphase cell, chromosomes showed a distinct movement back to the plate. When ATP was added to the PM + taxol after 7 min, the three cells behaved somewhat differently. The very early anaphase cell showed no reactivation; the mid-anaphase cell displayed chromosome movement to the poles and in the cell intermediate between these states, some chromosomes moved to the pole. These interesting preliminary results will be repeated when more taxol becomes available.

Stability of Microtubules in Permeabilized Cells

The stability of the interphase and mitotic MT systems under these experimental conditions was investigated by fixing and staining cells with fluorescent antitubulin. These experiments were conducted mostly on the PtK cultures.

MTs were relatively little affected within the first 7–9 min of permeabilization although some breakdown became obvious as time in the PM increased further. However, asters particularly in metaphase cells, consistently appeared larger than normal (compare Fig. 12, *a* and *c*); a polyploid cell (Fig. 12 *b*) shows this astral growth clearly. Similar results

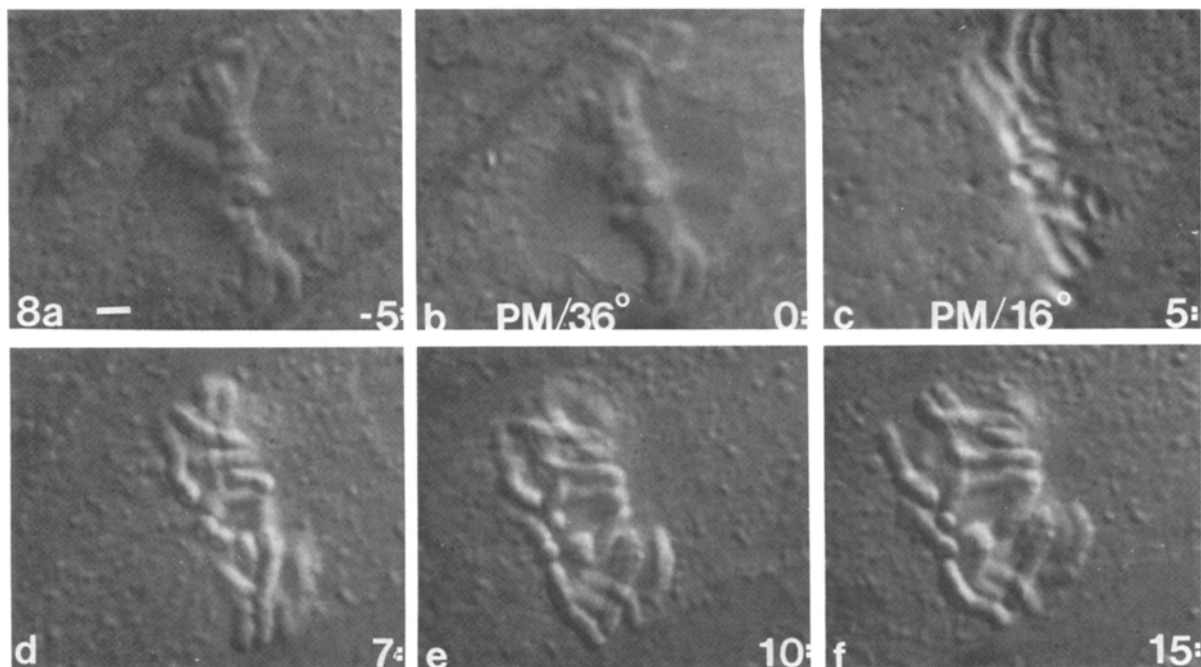


Figure 8. Reactivation of anaphase A by cool PM. (a) Metaphase. (b) Permeabilization at 36°C . (c) Anaphase A stopped, just before cool PM added; $T = 5$ min. (d-f) Movement of chromosomes in cool PM. Bar, 1 μm .

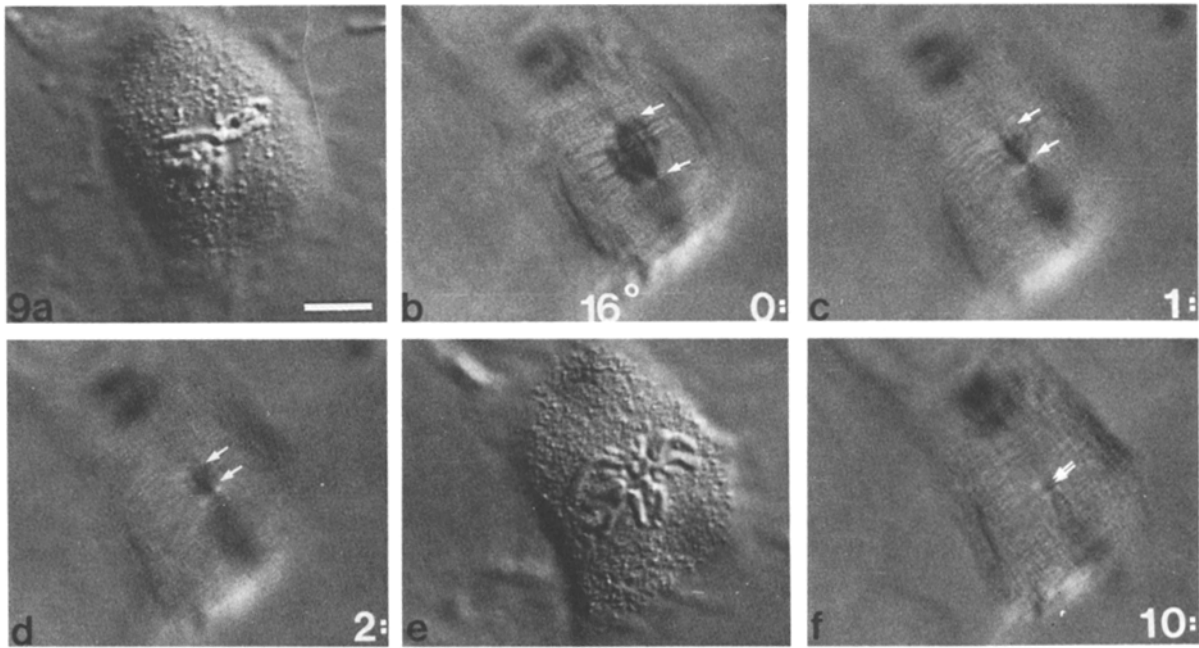


Figure 9. Control live cell, live under polarization optics. (a) Metaphase at 36°C. (b-f) Loss of spindle birefringence upon cooling to 16°C; arrows denote poles. Upon warming, these spindles reform. Bar, 10 μ M.

were obtained with cells treated with DNP/DOG and then permeabilized while still in DNP/DOG; astral growth in the PM + DNP/DOG was more obvious than in PM alone. Our impression was that DNP/DOG had a slight stabilizing effect on MT systems (as is also suggested by the slightly low rate of reactivated movement in ATP/DNP; see Table I). Cells treated with PM (6 min) and then 1 mM ATP in the PM (5 min) also showed relatively normal interphase and mitotic

MT arrays (Fig. 12 d), but anaphase kinetochore fibers were always short.

When cells were permeabilized and then treated with Ca^{++} in the PM, most interphase MT cytoskeletons were clearly present even after 15 min; in spindles, kinetochore MTs were short (at metaphase) or non-existent (late anaphase/telophase). Varying the proportions of Ca^{++} /EGTA did not seem to make much difference (Fig. 12, e-g). Again,

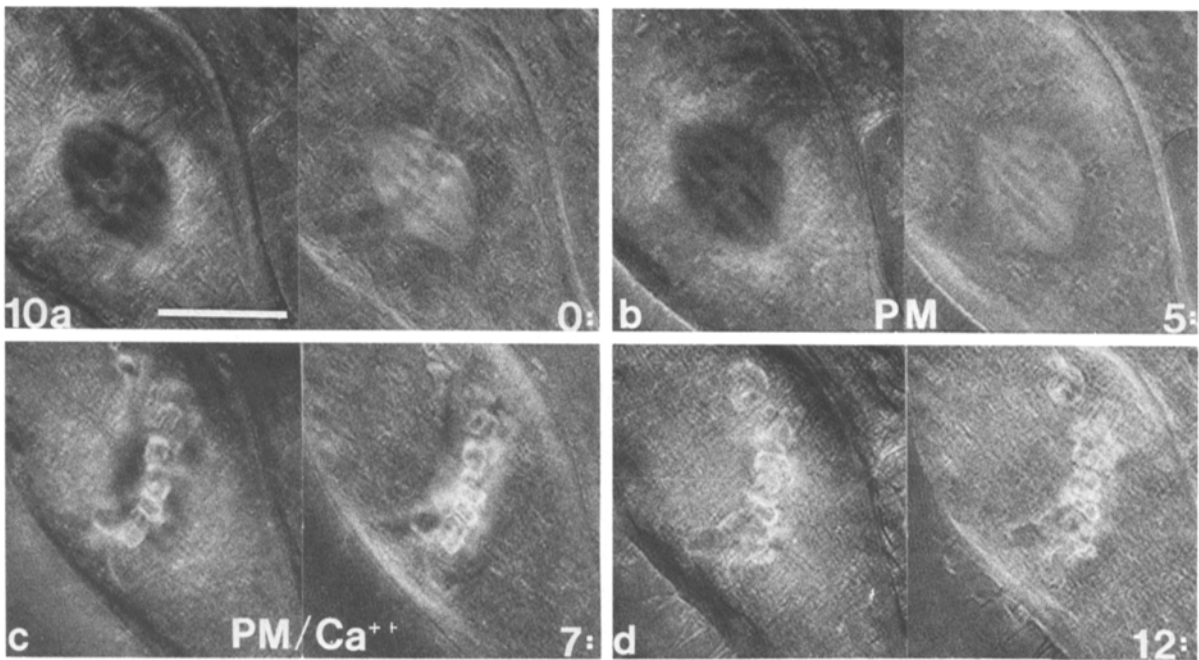


Figure 10. Control permeabilized cell, polarization optics. (a) Metaphase, just before permeabilization. (b) Spindle 5 min after permeabilization, just before addition of PM + Ca^{++} . (c and d) Loss of spindle birefringence in PM + Ca^{++} . Bar, 10 μ M.

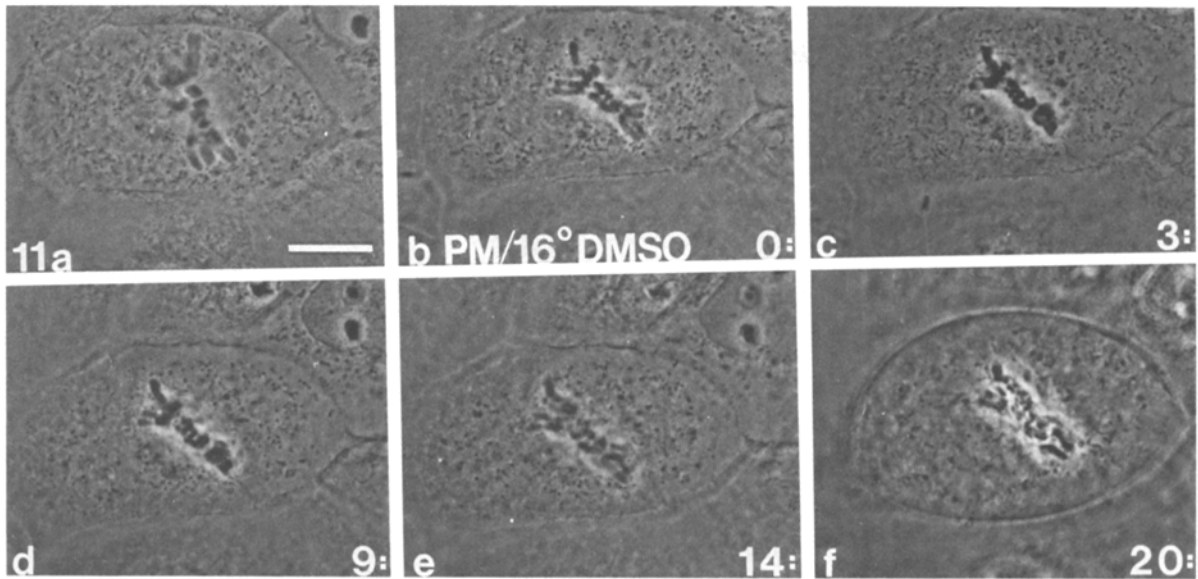


Figure 11. Inhibition of chromosome reactivation by MT stabilization. (a) Metaphase. (b) Permeabilization with cool PM + DMSO; (c-f) No reactivation of anaphase A (trace of separation has probably occurred by T = 14 min). Bar, 10 μ M.

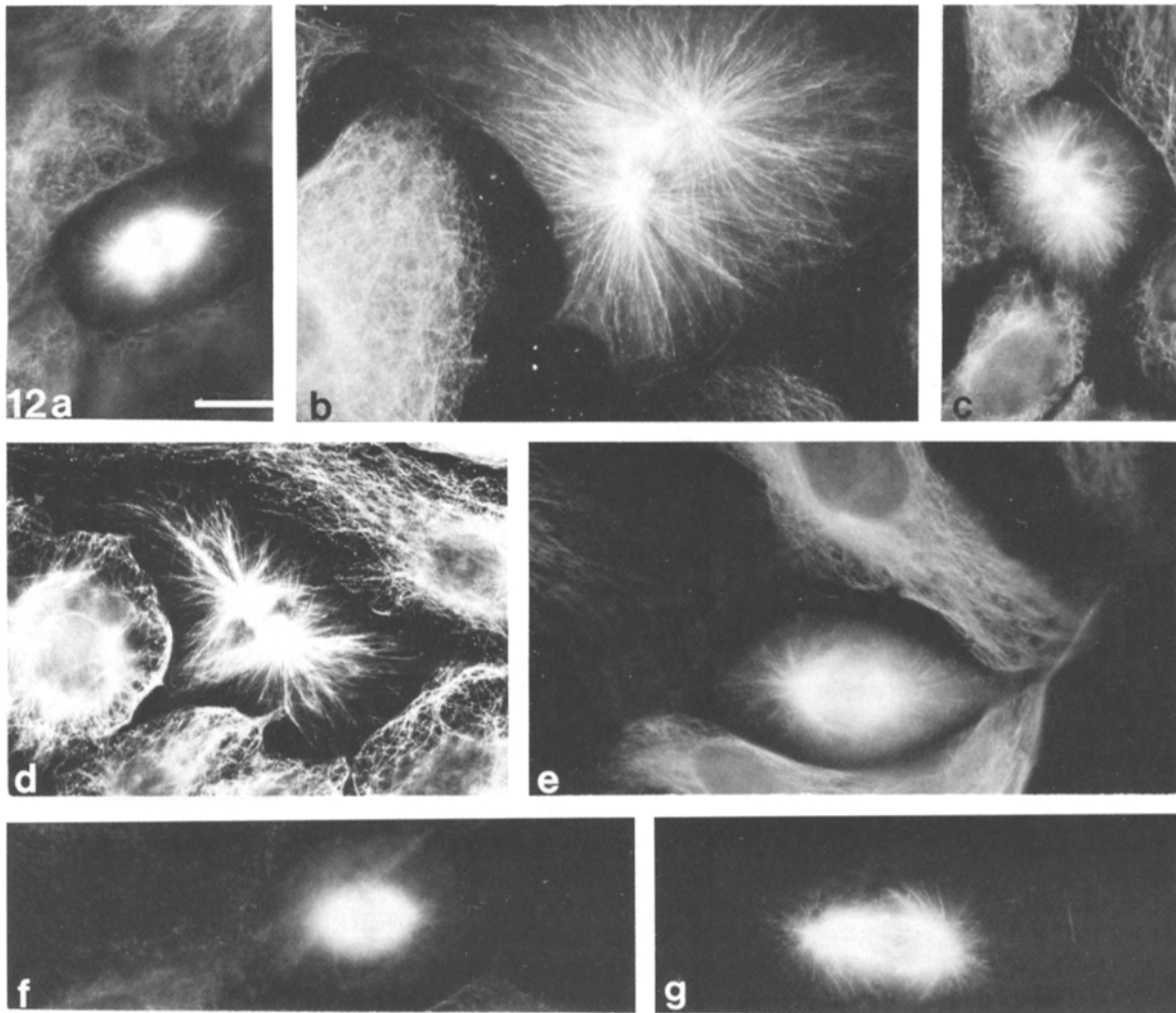


Figure 12. Fixed cells stained with antitubulin. (a) Normal metaphase from fixed cell. (b and c) Cells permeabilized 5 min before fixation. Asters typically larger than control; b is of a polyploid cell with larger than normal spindle showing marked astral growth. (d) Cell permeabilized for 5 min, then treated with PM + ATP, 5 min.; metaphase spindle still large. (e) Cell permeabilized (5 min), then treated with PM + Ca^{++} (5 mM); this treatment, which reactivates anaphase A, does not significantly break down most spindle or interphase MTs. (f and g) as for e but g was treated with 5 mM Ca^{++} in PM containing 1 mM EGTA (instead of the usual 10 mM EGTA). The metaphase spindles are not noticeably different in spite of the wide difference in free Ca^{++} . Bar, 10 μ M.

our impression was that some growth of asters often occurred within the first 5–10 min if 1 mM Ca^{++} was used in the PM. We checked this result by varying the proportions of Ca^{++} and EGTA in the PM. Coverslips of cells were treated 10 min with PM containing 1 mM of Ca^{++} , but the EGTA was varied between 10, 5, 2.5, 1, and 0 mM. In a second series, the Ca^{++} was increased to 10 mM while the level of EGTA was adjusted to 10, 5, 1, and 0 mM. The results were consistent: in both experiments, MT breakdown in interphase and mitotic cells was increasingly obvious as the Ca^{++} levels increased. Each coverslip showed a few interphase cells almost devoid of MTs; in the considerable majority of cells, interphase and mitotic MT arrays were present and well formed, although as the calcium level increased astral MTs were short to non-existent. Even when no EGTA was present, diminished MT arrays were present in most cells. Thus, while the concentration of free Ca^{++} present in PM containing EGTA is complex to calculate (Steinhardt et al., 1977), varying this concentration within wide limits does not greatly affect the MT systems in most permeabilized cells. Furthermore, kinetochore MTs were noticeably more sensitive than most other MTs to these manipulations. With increasing periods in PM, or with higher concentrations of digitonin in the PM, breakdown of all MT systems became widespread in PM containing 1 mM Ca^{++} . For comparison, PHEM-extracted MT cytoskeletons which are normally relatively stable over 10–15 min (see Spurck et al., 1986a) were rapidly broken down by 1 mM Ca^{++} .

Because PtK cells divide successfully at 20–25°C (Rieder, 1981), near the temperature of our cold flush, we checked the effects of low temperatures on our PtK cells. Live and per-

meabilized cells were chilled to several temperatures (18, 14, 10, 4°C) and then fixed and stained with antitubulin; the duration of chilling was standardized at 10 min, a midpoint in many of our reactivation protocols. In live cells, most interphase MTs are stable after 10 min of chilling to about 14°C. By 10°C, marked diminution of MTs was evident although the response of different cells varied considerably; some MTs were present in many live cells even at 4°C. In mitotic cells, astral MTs were markedly sensitive to lowered temperatures. Prophase spindles were tiny or non-existent; metaphase spindles were small and almost devoid of astral MTs at 18°C or below, as anticipated from observation of live cells *in vivo*; however, even at 14 and 10°C, thin, usually short kinetochore fibers were present and they were still detectable at 4°C. Anaphase cells had very short kinetochore fibers but interzonal MTs were evident, even at 14 and 10°C. The interzonal MTs and midbody of telophase cells were quite stable as expected, but the enlarged asters typical of these stages was increasingly destroyed by lowered temperatures. Similar results were obtained with permeabilized cells (Fig. 13). Interestingly, astral MTs appeared less effected than their counterparts in chilled, live cells; kinetochore fibers were, as anticipated, always shortened.

Two coverslips were treated with PM + taxol for 10 min; one was subsequently treated with PM + taxol + ATP for an additional 10 min. Both were fixed and stained as normal (not shown). The asters in the first coverslip were dense and large as expected, and some mitotic cells showed small, dense mini-asters as well. The second coverslip appeared much the same as the first but a subjective comparison suggested that some MT disassembly may have occurred. Ana-

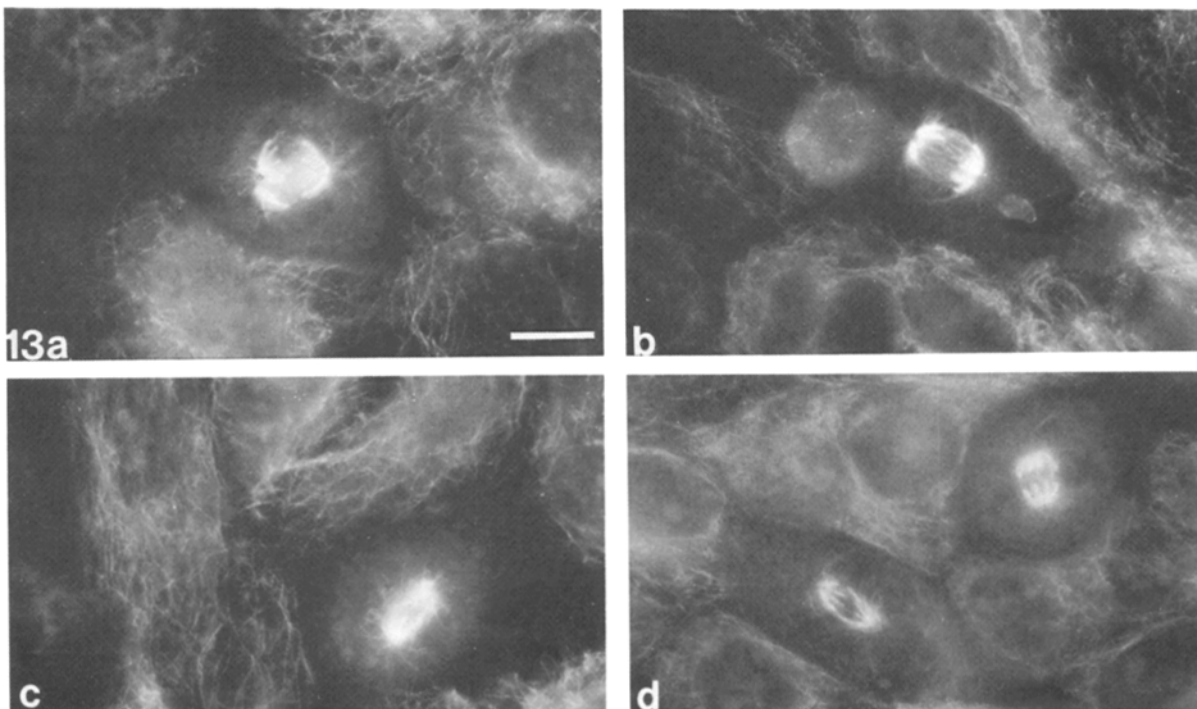


Figure 13. Cells permeabilized (5 min) and then chilled (5 min) before fixation. (a and b) Metaphase, anaphase cell chilled to 14°C; spindles diminished but kinetochore fibers visible. (c and d) As for a and b, but chilling was at 10°C; spindles small, but kinetochore fibers remain in metaphase cells. Bar, 10 μM .

phase cells had short kinetochore fibers, but, of course, it is impossible to know what stage these cells were in when first permeabilized.

Rates of Chromosome Movements

The results of these experiments are summarized in Fig. 14 and Table I. Chromosome movement was measured as the average distance between the sets of approximately parallel kinetochores (the poles could not be clearly distinguished and so pole-to-kinetochore measurements were not used). Each curve in Fig. 14 is from one representative cell undergoing the various treatments; Table I presents the average values for rates of anaphase A obtained from several such cells for each treatment. As mentioned above, there may have been some contribution of spindle elongation (anaphase B) to this measured separation, particularly in the control cells, so the rates are only approximate.

Anaphase B and Cleavage

We did not specifically study anaphase B or cleavage in this work. Rarely, we observed indications of anaphase B or a trace of cleavage. However, if we allowed chromosomes to reach the poles before permeabilization, then the subsequent addition of ATP in PM generated some anaphase B movement (top cell in Fig. 3); on some occasions, partial cleavage also occurred.

Discussion

Isolated spindles (Zimmerman and Forer, 1981; Salmon, 1982), rarely show mitotic movement (e.g., Sakai et al.,

1979; Sakai, 1978). Using less drastic permeabilization (Cande et al., 1974), chromosome movement has been reactivated and experimentally studied (reviewed by Snyder, 1981; Cande, 1982b). However, the observations have proved difficult to reproduce and interpret. We believe there are two problems in investigating anaphase A using permeabilized cell models. The first is technical, that of improving the experimental protocols sufficiently so that they are effective and reproducible. The second is conceptual; any investigation of the motor that powers chromosome movement for anaphase A is bound to be frustrating if such movement is consequent upon the release of chemical or physical energy stored in the spindle structure.

Permeabilization Techniques

With generous advice from Dr. M. Rozdzial (Rozdzial and Haimo, 1986), we have developed a reliable and reproducible permeabilization protocol. By waiting for 4-7 min after permeabilization (well after all cellular and chromosome motion had stopped) before attempting reactivation, we avoided the possibility that we were observing chromosome motion continuing in unaffected or poorly permeabilized cells. Furthermore, Rozdzial and Haimo (1986) found that mitochondria may remain functional (producing ATP) briefly after gentle permeabilization. We found that chromosome movement usually continued briefly after permeabilization, and we believe that the cessation of all movement marks the depletion of available ATP, as appears the case in live cells treated with metabolic inhibitors (Spurck et al., 1986a, b); the concurrent loss of small ions and metabolites might also occur. We (Spurck et al., 1986a, b) and others (e.g., Stanley et al., 1980) have found that cells contain a residual level of

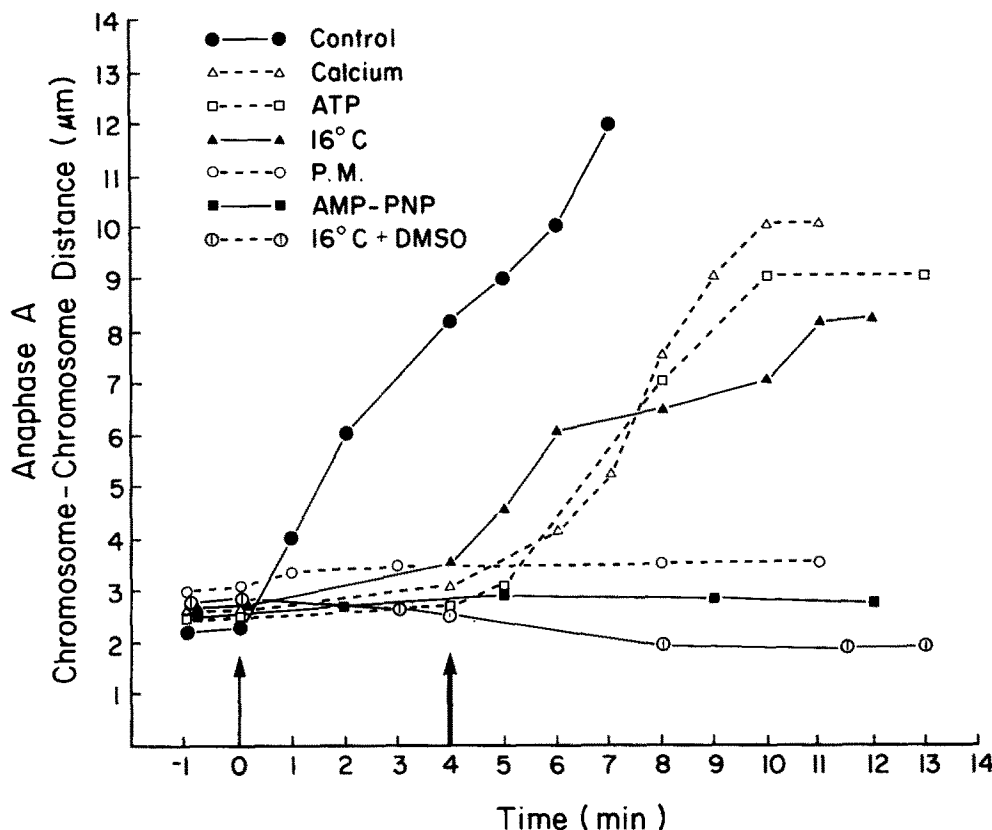


Figure 14. Distance between sets of daughter kinetochores plotted against time in seven cells treated different ways. Anaphase initiated at T = 0 min; treatments initiated at large arrow (T = 4 min). Bar, 10 µM.

ATP even after quite long exposures to metabolic inhibitors. There is no evidence that this ATP is available, for example, to motility systems since the cells remain completely quiescent and die after ~15 min in DNP/DOG. Nevertheless, the existence of this apparently unavailable pool of ATP must not be forgotten; whether the pool still remains in permeabilized cells is unknown.

We followed the influx of Hoechst dye into permeabilized cells (reviewed in Cande, 1982*b*), and additionally, the efflux of labels microinjected into the cell before permeabilization. At the low (0.0005%) concentrations of digitonin used, small molecules and ions can rapidly diffuse in or out of the rat hepatocyte while proteins are retained (Fiskum et al., 1980), without greatly affecting mitochondrial calcium uptake or release (Murphy et al., 1980). Increasing digitonin concentrations and time in the PM presumably led to an increasing leakage of proteins and a corresponding loss of our ability to reactivate spindles. Cande (1982*b*, 1987) likewise found that increasing extraction of the permeabilized cell significantly decreases its ability to display reactivated anaphase.

Energy Requirements for Mitosis in Live Diatoms and Conventional Cells

By treating diatoms with DNP, we (Pickett-Heaps, 1982; Pickett-Heaps and Spurck, 1982) concluded that polar movement of chromosomes is different from antipolar movement during prometaphase oscillations, and that the latter, but not the former, requires ATP. Assuming that polar movement at prometaphase and anaphase are the same, we suggested that both might be due to energy elastically stored in a structural matrix, the collar, that extends between kinetochores and the pole (reviewed in Pickett-Heaps et al., 1984*b*). Testing this hypothesis for anaphase A in diatoms is difficult because the chromosomes in many species are stretched almost to the poles in metaphase (e.g. Pickett-Heaps et al., 1980). In *Suriella* (Pickett-Heaps et al., 1984*a*) where this does not occur, we have demonstrated that anaphase B, but not anaphase A, is stopped by DNP (Cohn, S. A., and J. D. Pickett-Heaps, manuscript in preparation). In contrast, ATP is probably needed *in vivo* for anaphase A in most conventional spindles. We (Spurck et al., 1986*a*) and Hepler and Palevitz (1986) have found that in live mammalian and higher plant cells, anaphase is completely and reversibly stopped by metabolic inhibitors.

We consider it unlikely that the different response of animal and diatom cells to inhibitors during anaphase A is due to diatoms having an unusual mechanism of chromosome motion. More relevant is the different structural relationship between kinetochores and MTs in the two spindle types. In diatoms, kinetochores are laterally associated with polar MTs, and slide along them during prometaphase and anaphase (Tippit et al., 1980). In contrast, by metaphase mammalian kinetochores have MTs terminating in them.

Reactivation of Spindles in Permeabilized Cells

Recent work on energy requirements for anaphase A in permeabilized cells is confusing (see the introduction). Cande and Wolniak (1978) and Cande (1979) showed that ATP is necessary for anaphase A—as we describe here—but later, Cande (1982*a*) stated that it is not needed for anaphase A, but is for anaphase B. The reasons for these differences are

not clear. However, Cande (personal communication) agrees that his first experiments were conducted under conditions where the calcium level was not well buffered and therefore likely under MT disassembly conditions that would permit anaphase A to continue.

In our PtK and cultured newt cells, mitosis and cytoplasmic activity ceases within ~2–3 min of permeabilization. Addition of ATP and GTP (1–10 mM) in the PM after 5–7 min causes anaphase to restart. We also demonstrate the need for ATP by first depleting live cells with DNP/DOG; after their chromosomes had stopped moving (Spurck et al., 1986*a*), we permeabilized them in PM + DNP/DOG. Reactivation of anaphase A followed addition of ATP to the PM + DNP/DOG. We are confident that in both live and permeabilized PtK and newt cells, ATP is needed for anaphase A. Since similar reactivation was achieved with 0.1 mM of the analog ATP- γ -S which supports phosphorylation but, as far as is known, not reactivation of cytoplasmic motility systems (Shimizu and Johnson, 1986), this requirement for ATP may be for phosphorylation rather than for an energy source.

The Role of ATP during Anaphase A

In our opinion, there are two likely ways ATP could be involved in anaphase A: (a) as the power source for an uncharacterized motility system; and (b) in disassembly of the kinetochore MTs. We will now examine these possibilities which are not mutually exclusive.

The origin of the force that moves anaphase chromosomes is unknown. This force exists continuously during metaphase as well as at anaphase; metaphase spindles often appear to be under considerable compression (e.g., LaFountain, 1972) and if the diatom spindle is severed by a microbeam, the poles collapse towards each other (e.g., Leslie and Pickett-Heaps, 1983). That this force is pulling individual chromosomes polewards even during metaphase is shown by inactivation of a single kinetochore by a microbeam (McNeill and Berns, 1981); that chromosome is immediately pulled to the pole to which the undamaged kinetochore is attached while the other chromosomes remain central. Inoue (1981) has demonstrated with live eggs of *Chaetopterus* that rapid disassembly of spindle fibers leaves chromosomes “stranded,” while slower disassembly will cause metaphase chromosomes to move to the cell cortex where one pole of the spindle is anchored. Similar observations were made by Fuseler (1975) and by Salmon (1975) who used slow and more rapid disassembly induced by high pressure. We (Spurck et al., 1986*a, b*) have emphasized how the poles of live metaphase cells are similarly drawn to the metaphase plate as the spindle fibers are broken down with anti-MT agents. In these situations, the live cells were not energy depleted. However, when isolated metaphase spindles from sea urchin eggs are exposed to micromolar Ca⁺⁺ levels, similar disassembly of spindle fibers is induced, often accompanied by shortening of the half spindle (Leslie et al., 1987) and movement of chromosomes if one end of those spindles had become attached to the glass slide (Salmon and Segall, 1980; Salmon, 1982). We conclude that the force-producing machinery evident in live cells could exist even in isolated spindles, and that it could function without an external source of energy.

The second alternative is that this need for ATP in anaphase A might be linked to MT disassembly. We and others (e.g., Bershadsky and Gelfand, 1981; Moskalewski et al.,

1980) find that ATP-depletion actually stabilizes MTs against the rapidly disruptive effects of anti-MT drugs such as nocodazole and colcemid. Hinz et al. (1986) have shown that in live diatoms, the normal, unidirectional disassembly of the half-spindles after mitosis is reversibly stopped by metabolic inhibitors. From these observations on ATP and MT disassembly, we (Spurck et al., 1986b) concluded that a role for ATP in conventional spindles might be in the disassembly of kinetochore MTs known to be required for anaphase A to progress when disassembly is the rate-limiting step (e.g. Fuseler, 1975; Salmon and Begg, 1980). This rationale explains why diatoms do not require ATP during anaphase A since chromosome-to-pole movement is accomplished by kinetochores sliding along polar MTs; disassembly is not necessary.

We have reactivated anaphase A with several high energy, hydrolyzable compounds (ATP, GTP, ATP- γ -S, GTP- γ -S); reactivation was not observed with the nonhydrolyzable analogue AMPPNP which also apparently inactivated the spindle, presumably by binding to some component. It might be argued that ATP- γ -S and GTP- γ -S could reactivate a spindle motile system. However, these and ATP and GTP are known to break down MT cytoskeletons (Spurck et al., 1986b). We have also reactivated anaphase A using two other protocols that disassemble MTs: perfusion with either PM containing Ca^{++} or with cold PM (16°C); under these conditions, the permeabilized cells were devoid of an external energy source. We conclude that the energy needed to move chromosomes during anaphase A must be stored (presumably elastically or possibly chemically; see below) in the spindle since we cannot imagine how cold treatment alone would be capable of reactivating a motility system in an apparently energy-depleted cell.

The Dynamic Equilibrium Model

Inoue and Sato (1967; also Inoue et al., 1975) proposed that the disassembly of spindle fibers might drive the movement of chromosomes polewards, a proposal often endorsed (e.g., Fuseler, 1975) and recently treated theoretically (Hill and Kirschner, 1983). The results we have obtained, especially from the cold and Ca^{++} experiments, are compatible with this possibility. We have discussed elsewhere why the kinetochore, besides capturing polar MTs (Tippit et al., 1980), might be actively involved in generating movement over these MTs while disassembling them at the kinetochore end (Pickett-Heaps et al., 1982, 1986; Mitchison et al., 1986). Thus, the kinetochore could be the agent in transforming this energy of disassembly into chromosome motion. While we consider this a reasonable possibility, we prefer the elastic model mentioned above for several reasons. For example, diatoms can move chromosomes polewards without MT disassembly or ATP (anaphase A continues in DNP: Cohn, S. A., and J. D. Pickett-Heaps, manuscript in preparation) and chromosome movement often displays elastic properties (e.g., the polewards force on a chromosome being proportional to its distance from the pole: Wise, 1978; Hays et al., 1982; see discussion in Pickett-Heaps et al., 1986). Our present experiments do not resolve this issue. Neither do we consider the elastic matrix model incompatible with the concept that the kinetochore might be an active participant in chromosome movement.

The Role of Calcium at Anaphase

Hepler and Palevitz (1986) disagree with our interpretation (Spurck et al., 1986a) concerning the role of ATP in kinetochore MT disassembly, speculating instead that ATP depletion may inhibit anaphase A via effects on membrane-localized ion transport systems in the spindle. They (e.g., Hepler, 1980; Wick and Hepler, 1980; Hepler and Wolniak, 1984) and others (e.g., Salmon and Segall, 1980) favor a role of these membranes in the localized release of Ca^{++} which in turn could selectively control the stability of kinetochore and other fibers.

While this attractive view has many proponents (Ratan and Shelanski, 1986), the concept has problems that have not yet been well addressed. For example, the specific membrane association with kinetochore MTs necessary to induce this selective disassembly is not present in the closed spindles characteristic of a majority of algae, fungi, protozoa—nor indeed is it in many conventional spindles. Hepler (1985) and Izant (1983) found that the rate of anaphase chromosome motion was unaffected in live cells whose Ca^{++} levels were disturbed artificially. More convincing is the recent evidence that calcium may act as the trigger that instigates anaphase (e.g., Wolniak and Bart, 1985a, b; Poenie et al., 1986). Poenie et al. (1986) find that the elevation of intracellular Ca^{++} that coincides with initiation of anaphase is transient and that intracellular levels return to normal during anaphase; they find no evidence for localized regions of high calcium being maintained during anaphase.

The present experiments cannot resolve this issue, but they indicate that MT disassembly can occur in the stabilizing environment of the EGTA-buffered PM in which the concentration of free calcium is very low. In permeabilized cells, this disassembly, induced by a variety of agents during anaphase A, is highly selective as it is in the normal live spindle: kinetochore MTs are shortened while other MTs may remain unchanged or even elongate. Such selectivity argues against the concept that localized calcium release controls MT disassembly since intermingled kinetochore, astral, and interzonal MTs should all be affected similarly and in permeabilized cells, the cytoplasmic Ca^{++} level is presumably uniformly buffered.

Stability of MTs in the Permeabilized Cells

Photomicrographs of permeabilized mitotic cells fixed and stained with antitubulin confirmed that astral and kinetochore MTs are stable in PM alone; the PM is designed as an MT-stabilizing buffer. When anaphase A was induced with ATP, kinetochore MTs shortened while there was a tendency for asters to grow; in contrast, both kinetochore and astral MTs soon shrank to almost nothing in PM plus Ca^{++} or in cold PM. In live cells too, kinetochore MTs, more stable than nonkinetochore MTs (reviewed by Rieder, 1982; Scarcello et al., 1986), are specifically shortened during anaphase A. We propose that the force acting on each fiber is the prime activator for ATP-mediated MT disassembly (Pickett-Heaps et al., 1986). This concept still applies in the present experiments since the spindle's force-producing machinery remains functional in permeabilized spindles and energy-depleted live spindles.

Many MTs in permeabilized mitotic and interphase cells remained intact in the Ca^{++} levels used to activate anaphase

(i.e., while kinetochore MTs shortened) in the absence of ATP. The stability of MTs in PM was checked by treating cells with varying concentrations of Ca⁺⁺ and EGTA in the PM; even with no EGTA, an appreciable population of MTs remained in 1 mM and even 10 mM Ca⁺⁺ although loss of MTs was obvious. As permeabilization became more extensive (i.e., as the detergent concentration and/or time of incubation in PM increased), so did this level of calcium show its expected effect in disassembling interphase and other spindle MTs (Salmon, 1982). Likewise, MT cytoskeletons from PHEM-extracted cells (as in Spurck et al., 1986b) were very sensitive to calcium. These changes may correlate with the increasing extraction of proteins that control MT stability and assembly/disassembly in vivo.

Taxol Experiments

Only a few experiments could be carried with taxol. In one cell permeabilized at mid-anaphase in PM + taxol, the chromosomes, after ceasing polar movement, reversed direction, moving back toward the metaphase plate. Bajer et al. (1982) found that promoting MT assembly with this drug causes a transient pushing force back to the equator in live cells. We also reactivated some chromosomal movement in permeabilized cells with taxol + ATP, depending on the stage reached in anaphase. Bajer et al. (1982) state that anaphase will continue after the initial anti-polar movement (see above) induced by taxol.

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