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CHROMOSOME MOVEMENT IN LYSED MITOTIC CELLS IS INHIBITED BY VANADATE

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ABSTRACT

Mitotic PtK_1 cells, lysed at anaphase into a carbowax 20 M Brij 58 solution, continue to move chromosomes toward the spindle poles and to move the spindle poles apart at 50% in vivo rates for 10 min. Chromosome movements can be blocked by adding metabolic inhibitors to the lysis medium and inhibition of movement can be reversed by adding ATP to the medium. Vanadate at micromolar levels reversibly inhibits dynein ATPase activity and movement of demembranated flagella and cilia. It does not affect glycerinated myofibril contraction or myosin ATPase activity at less than millimolar concentrations. Vanadate at 10–100 μM reversibly inhibits anaphase movement of chromosomes and spindle elongation. After lysis in vanadate, spindles lose their fusiform appearance and become more barrel shaped. In vitro microtubule polymerization is insensitive to vanadate.

KEY WORDS dynein · anaphase · cilium myosin · lysed cell · mitosis · vanadate

The mitotic spindle is responsible for the equipartition of chromosomes during cell division. At anaphase the sister chromatids separate, the chromosomes move to the spindle poles, and the spindle elongates. Although these events have been described by light and electron microscopy, the mechanism by which the spindle moves chromosomes is not known. It is generally agreed that the fibrous components of the spindle are the mechanochemical elements that generate the forces necessary for chromosome movement. Microtubules are found attached to the chromosomes at kinetochores and radiating out from each pole to form the overall framework of the spindle (14, 16). Actin microfilaments are also found in the spindle, but their role during anaphase is not understood (4). It has been suggested that shear forces ample for chromosome movement are generated within the spindle by microtubule-microtubule interactions mediated by dynein cross bridges analogous to the mechanochemical system found in cilia and flagella (reviewed in reference 16). Polymerization and depolymerization of microtubules may also play a role in force generation or in regulating the rates at which chromosomes move during anaphase (9, 16). Alternatively, actomyosin or an actomyosin-microtubule complex may be responsible for some aspects of chromosome movement (4, 16).

Through the use of demembranated cell model systems the interactions between proteins that generate movement in flagella and in muscles are beginning to be understood. In vitro studies of mitosis using lysed cell models may also provide clues to the underlying mechanism of chromosome movement (2, 14, 19). We report here that anaphase chromosome movement in lysed PtK_1 cells is an ATP-dependent process. Spindle structure is stabilized after lysis in the presence of calcium chelating buffer systems and carbowax 20 M and mild lysis is achieved by using appropriate concentrations of the nonionic detergent Brij 58 (2).

Recently, Cantley et al. (3) reported that vanadate in the V^+ oxidation state is an inhibitor of the oubain-sensitive Na^+/K^+ ATPase, but that other ATPases such as myosin, the F_1 ATPase of mitochondria, and the Ca^{++} ATPase from sarcoplasmic reticulum are unaffected except at very high concentrations. We have found that vanadate

in the V^+ oxidation state is a potent inhibitor of the flagellar ATPase dynein and of reactivated beat in demembranated cilia and flagella. During the course of this investigation, similar results were described by Gibbons et al. (6) and by Kobayashi et al. (11). In this paper, we report that vanadate is also a potent inhibitor of chromosome movement in lysed cell models of dividing mammalian tissue culture cells.

MATERIALS AND METHODS

Materials

Sodium orthovanadate was obtained from Accurate Chemical and Scientific Corp., Hicksville, N. Y. Vanadate-free ATP prepared from yeast was obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Carbowax 20 M (polyethylene glycol, mol wt 20,000) was obtained from Sigma Chemical Co., St. Louis, Mo., and Brij 58 was a gift of the Atlas Chemical Industries, Inc., Wilmington, Del. Hog neurotubulin, prepared as described previously (21), was resuspended in 100 mM piperazine-N-N'-bis(2-ethane sulfonic acid (PIPES), pH 6.94, 1 mM $MgSO_4$, 1 mM GTP and assembly of microtubules was monitored by following changes in light scattering at 395 nm (5). Sperm were obtained from the sea urchin *Strongylocentrotus purpuratus* by injection with 0.5 M KCl. Demembranated sea urchin sperm were prepared and reactivated as described by Brokaw et al. (1). Bracken fern (*Pteridium aquilinum*) spermatozoids have 32 cilia clustered at one end of the cell and the cilia will continue beating normally for hours after the cells are stuck to polylysine coated glass slides at their proximal end (23). Cells were demembranated and reactivated as described by Wolniak and Cande (23).

ATPase Preparations and Assay

Heavy meromyosin (HMM) was prepared from rabbit skeletal myosin by the method of Lowey and Cohen (12) and actin was prepared by the method of Spudich and Watt (22). *Dictyostelium* myosin was a gift from Dr. James Spudich (Stanford University). Mg^{++} activated ATPase activity was measured in a buffer containing 4 mM $MgCl_2$, 0.01 M imidazole, pH 7.0, 1 mM ATP, and actin activated ATPases were run in a similar buffer containing 24.3 μg/ml HMM or myosin and 71 μg/ml actin. Axonemes were prepared from *S. purpuratus* sperm by the method of Gibbons and Fronk (7) with the modification that 1 mM $CaCl_2$ was added to all solutions and 10 mM 4-[2-hydroxyethyl]-1-piperazine propane sulfonic acid (EPPS), pH 8.0, was used as a buffer. The axonemes were extracted for 5 min at 4°C in 0.6 M KCl by the method of Gibbons et al. (6). The ATPase activity of the extracts was measured as above in an assay containing 0.15 M KCl, 2 mM $MgSO_4$, 0.5 mM EDTA, 10 mM EPPS, pH 8, 1 mM dithiothreitol (DTT), and 1 mM ATP. All experiments were run at 25°C. Inorganic phosphate production was measured by the method described in Pollard and Korn (18).

Tissue Culture Cells and Cell Lysis

PtK_1 cells were used in all experiments and were maintained and handled for light microscopy as described previously (2, 21). Coverslips were mounted on slides with coverslip fragments as spacers. Cells entering anaphase were lysed by flushing solutions under the coverslip in a two-step procedure: Step 1 medium contained 90 mM PIPES, pH 6.9, 0.05% Brij 58, 6 mg/ml bovine serum albumin, 0.1 mM DTT, 1 mM ethyleneglycol-bis-(β-Amino-ethyl ether)N,N'-tetraacetic acid (EGTA), 1.25 mM ATP, 2.25 mM $MgSO_4$. Step 2, which followed 60 s after step 1, used a similar medium that included in addition 2.5% carbowax 20 M and 0.1% Brij 58. All experiments were run at 35°C.

Films were made for studying chromosome movement using Zeiss Nomarski optics and an Opti Quip (Opti Quip Inc., Highland Mills, N. Y.) 16 mm cine time lapse apparatus. Exposures of 0.5 s duration were made at a rate of 10 frames/min. Rates of movement were estimated by measuring the slopes of the graphs drawn of chromosome-chromosome and pole-pole distances from the moment of addition of the second step medium to the position of maximum displacement of chromosomes or poles. The rate of chromosome-to-pole movement was calculated as one half the difference between the rates of chromosome separation and spindle elongation. Measurements of spindle birefringence were made using a Zeiss polarization microscope (21).

RESULTS

Effect of Vanadate on Dynein and Myosin

Dynein extracts prepared by brief, high salt extraction of demembranated sea urchin axonemes are 5–10% dynein and 80% tubulin as determined by densitometric scans of sodium dodecyl sulfate polyacrylamide gels (Cande, unpublished data). Unlike extracts prepared by Gibbons et al. (6), these preparations contain multiple dynein bands including dynein band 2. The ATPase activity of these extracts is inhibited 60% by 1 μM vanadate and is 95% inhibited by 100 μM vanadate (Fig. 1). As has been reported previously (3, 6, 11), norepinephrine is effective in preventing the inhibition of ATPase activity when added with vanadate (Fig. 1).

Vanadate has no inhibitory effects on the Mg^{++}-activated or actin-activated ATPase activity of *Dictyostelium* myosin or rabbit skeletal HMM, unless 1,000 μM vanadate is included in the incubation mixtures (Fig. 1). We found a slight

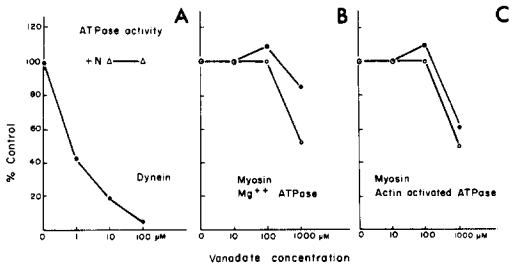

FIGURE 1 Inhibition of ATPase activity by vanadate. (*A*) Dynein extracts are 10% dynein as described in Materials and Methods (●, △). Specific activity in absence of vanadate or in presence of vanadate and 2.5 mM norepinephrine (*N*) is 0.165 μmol Pi × mg^{-1} total protein × min^{-1}. (*B*) Mg^{++} activated *Dictyostelium* myosin (○) or rabbit skeletal heavy meromyosin (●). Specific activities in absence of vanadate are 0.011 and 0.018 μmol Pi × mg^{-1} × min^{-1}, respectively. (*C*) Actin activated *Dictyostelium* myosin (○) or rabbit skeletal heavy meromyosin (●). Specific activities in absence of vanadate are 0.18 and 0.24 μmol Pi × mg^{-1} × min^{-1}, respectively.

but consistent stimulation of HMM ATPase activity in the presence of 100 μM vanadate. These results demonstrate that myosins from several sources are insensitive to vanadate over a concentration range that completely inhibits dynein ATPase activity.

Effect of Vanadate on Demembranated Cell Models

Vanadate reversibly inhibits the flagellar beat of demembranated *S. purpuratus* sperm or the ciliary beat of partially demembranated bracken fern spermatozoids. Intact sperm or spermatozoids are insensitive to millimolar vanadate; however, the detergent-treated cells are inhibited even at micromolar concentrations. Ciliary beat is inhibited in seconds by 10–100 μM vanadate, although cilia will continue beating for 1 min in 1 μM vanadate before inhibition occurs. After inhibition by vanadate, the cilia or flagella are straight or slightly curved.

Beat can be restored to the demembranated fern cilia by dilution of the suspension with additional reactivation medium, so that the vanadate concentration falls well below 1 μM. Ciliary beat resumes within seconds of dilution. The inhibition of flagellar and ciliary beat by vanadate can be reversed by adding norepinephrine to the vanadate containing medium. Cilia or flagella when reactivated in norepinephrine plus vanadate display normal beating. This result is consistent with reports that norepinephrine reduces vanadate to the inactive IV^+ oxidation state and may chelate the IV^+ salt (3, 6).

Glycerinated rabbit myofibrils incubated for 10–30 min in 1–5 mM vanadate will contract when ATP is added to the preparation. No inhibition of myofibril contraction was ever observed.

Chromosome Movement in Lysed Mitotic Cells

PtK_1 cells after lysis are permeable to small molecules such as calcium and the dye erythrosine B and to proteins such as tubulin and rhodamine-labeled immunoglobulin (reference 2; manuscript in preparation). Routinely, chromosome movements continue for 10 min after lysis in ATP-containing medium, and in 70% of the experiments run, chromosome separation in excess of 5 μm is observed. During the first 10 min of anaphase in unlysed cells, chromosomes and poles move apart at rates approaching 2 μm/min. Chromosome separation rates in lysed cells are maintained at 55% in vivo rates, and spindle elongation occurs at 40% in vivo rates during the first 8–10 min after lysis (Tables I and II; Fig. 2).

Nucleotide hydrolysis is required for chromosome movement. If no ATP is included in the lysis medium, some chromosome movement occurs after lysis; however, little or no movement occurs if uncouplers of oxidative phosphorylation such as 2,4-dinitrophenol (DNP) (Table I) or carbonyl cyanide, m-chlorophenylhydrazone (CCCP) (Fig. 2) are included in the lysis medium. This inhibition of movement can be countered if ATP is added at the time of cell lysis; however, the nonhydrolyzable nucleotide analogue adenyl(β,γ-methylen)-diphosphate (AMPPCP) is ineffective in restoring chromosome movements.

Anaphase Movements are Inhibited by Vanadate

Vanadate inhibits poleward movement of chromosomes and spindle elongation when the salt is included in the lysis medium (Table II; Figs. 2 and 3). Inhibition is rapid and a reduction in the rate of chromosome separation is observed during the first 2 min after lysis. Chromosome separation is inhibited by 50% in 10 μM vanadate, and inhibition is essentially complete in 100 μM vanadate. Chromosome-to-pole movements and spindle elongation have a similar sensitivity to vanadate (Table II). Cells lysed late in anaphase display

TABLE I

Effect of Metabolic Inhibitors on Chromosome Movements

Inhibitors	Nucleotide	Rates of movement (μm/min)		
		Chromosome separation	Spindle elongation	Chromosome to pole movement
10^{-4} M DNP* 20 mM Arsenate	1.25 mM AMPPCP	0.24	0.14	0.03
10^{-4} M DNP* 20 mM Arsenate	1.25 mM ATP	0.85	0.50	0.23

* Average of three separate experiments.

TABLE II

Effect of Vanadate on Chromosome Movements

Treatment		Rates of movement (μm/min)		
Vanadate concentration*	No. of experiments	Chromosome separation	Spindle elongation	Chromosome to pole movement
None	11	1.07 ± 0.01‡	0.71 ± 0.09	0.18 ± 0.09
10 μM	8	0.55 ± 0.01	0.42 ± 0.01	0.05 ± 0.02
100 μM	9	0.36 ± 0.06	0.23 ± 0.03	0.02 ± 0.02
After reversal	3	1.04	0.61	0.22

* All experiments were run in presence of 1.25 mM ATP. For reversal experiments, after 4 min in 100 μM vanadate, a fresh solution containing 2.5 mM norepinephrine was added to the preparation.
‡ Variance.

only spindle elongation since the chromosomes have already approached the spindle poles. These movements are also inhibited by vanadate.

The inhibitory effects of vanadate are reversible. Chromosome-to-pole movements and spindle elongation rates will increase several fold if the vanadate solution is replaced with another medium containing norepinephrine (Table II; Figs. 2 and 3). Although vanadate reversals have been successfully repeated even after 4 min exposure to vanadate, we have not been able to restore chromosome movement if norepinephrine is left out of the wash step.

Anaphase movements in unlysed PtK_1 cells are not affected by 1 mM vanadate if it is added to the culture medium during metaphase. Cells grown in 100 μM vanadate for 4 h still undergo mitosis.

Effects of Vanadate on In Vitro Tubulin Polymerization and Depolymerization

The rate and extent of tubulin polymerization is not affected by millimolar vanadate in the polymerization medium (Fig. 4). Microtubules formed in the presence of vanadate are cold-labile and calcium-sensitive and cannot be distinguished ultrastructurally from control preparations.

Effects of Vanadate on Spindle Birefringence

Spindle birefringence after lysis is maintained at in vivo levels for 8–10 min before it begins to fade. Spindle birefringence is calcium-labile and will disappear in 20 s when 5 mM calcium is added to the lysis medium (2).

Vanadate does not quantitatively alter the level of spindle birefringence after lysis, nor does it alter the stability of the spindle (Fig. 5). However, vanadate does subtly alter the overall pattern of spindle birefringence. After several minutes the spindles lose their fusiform appearance and assume a barrel shape. The chromosomal fibers no longer bend in towards the spindle poles but begin to straighten out (Fig. 5). During this process the spindle does not shrink but retains its original length. In some cells the chromosomal fibers become very diffuse and individual fibers take on a broad, fan shape spreading out from each chromosome. These changes have been observed both in metaphase and anaphase cells lysed in vanadate

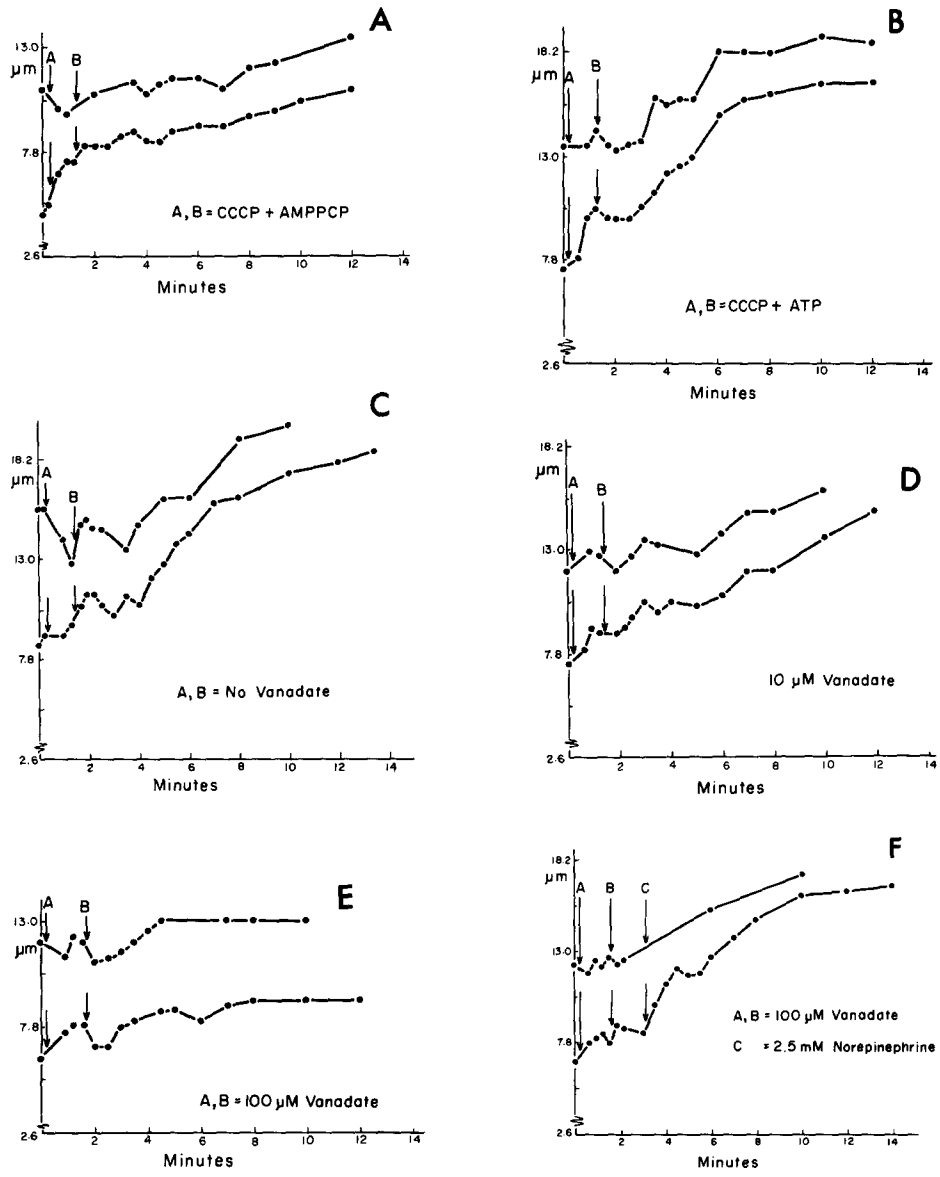

FIGURE 2 *A–F*. Separation of sister chromatids (lower lines) and spindle poles (upper lines) after lysis. Lysis medium added at A and B as described in the text. All solutions contained 1.25 mM ATP except *A*, which contained 1.25 mM AMPPCP. In *A* and *B* the lysis medium contained in addition 10^{-5} M CCCP, in *C*, no inhibitors, in *D*, 10 μM vanadate, in *E*, 100 μM vanadate. In *F* a cell is lysed in 100 μM vanadate at A and B, then this solution is replaced by solution C, which contains 2.5 mM norepinephrine.

but have not been observed under any other lysis conditions including lysis in the presence of metabolic inhibitors or 10 mM EGTA. Upon addition of norepinephrine the spindles revert back to their original shape.

DISCUSSION

In this report, we demonstrate that chromosome movement during anaphase is an ATP-dependent process that is sensitive to vanadate in the V^+ oxidation state. Vanadate blocks ciliary beat in demembranated cells by inhibiting dynein ATPase activity; it does not block glyerinated myofibril contraction, nor does it inhibit cytoplasmic or skeletal muscle myosin ATPase activity except at millimolar concentrations. With regard to sensitiv-

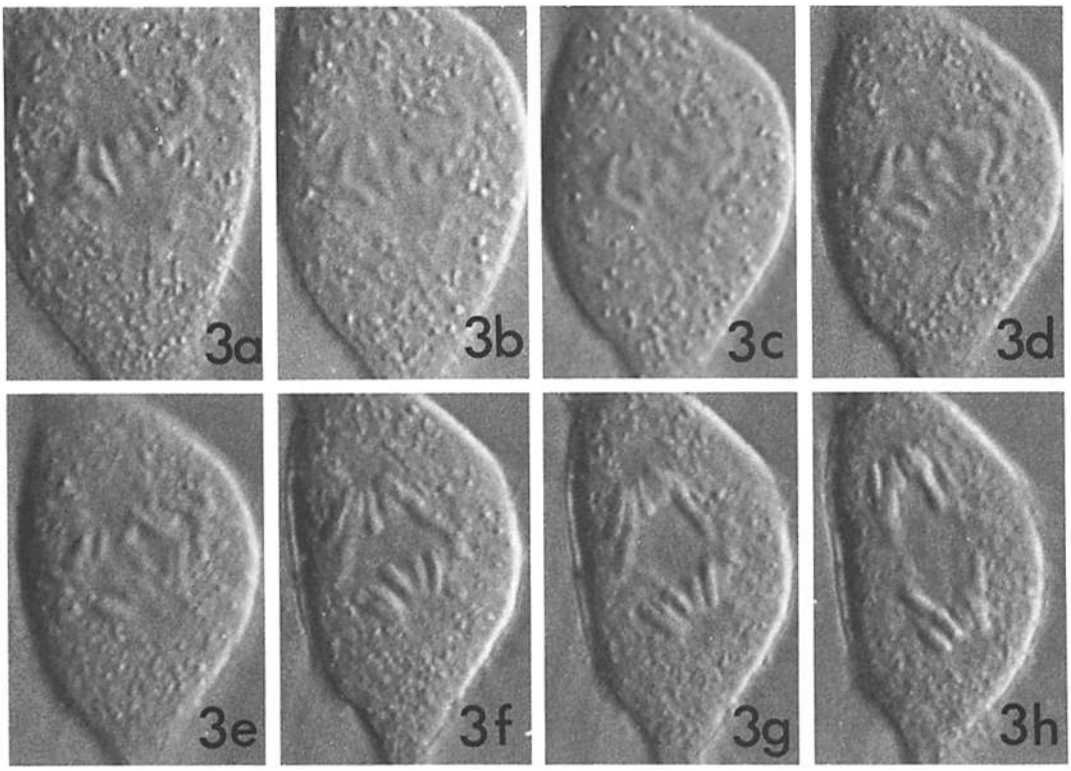

FIGURE 3 *A-H*. Micrographs of anaphase in lysed PtK_1 cells demonstrating reversible inhibition of chromosome separation and spindle elongation by 100 μM vanadate. After 4 min in vanadate, a solution containing 2.5 mM norepinephrine is added. *a*: 0.1 min before lysis, *b*: 1 min after lysis, *c*: 2 min, *d*: 3.8 min, *e*: 4.2 min, *f*: 6 min, *g*: 8 min, *h*: 12.5 min after lysis. × 2000.

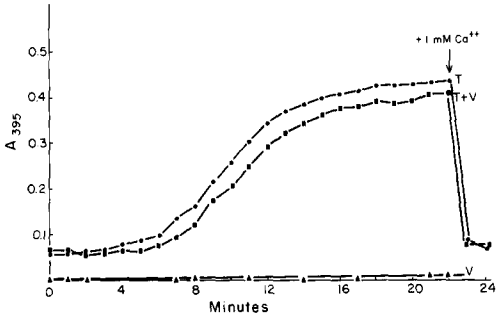

FIGURE 4 Extracts containing 5 mg/ml tubulin (T), tubulin and 1 mM vanadate ($T + V$), or buffer solutions and vanadate (V) are warmed to 37°C and changes in turbidity are monitored spectrophotometrically. At the end of the experiment, $CaCl_2$ to 1 mM is added to each extract.

ity to vanadate and to reversal of inhibition in the presence of norepinephrine, chromosome movement and spindle elongation resemble those in a cilium more than in a muscle.

The basis of the requirement for nucleotide triphosphates such as ATP for mitosis is not known. Mechanochemical ATPases such as dynein and myosin have been postulated to play a role in force generation for chromosome movement and spindle elongation (reviewed in references 4, 14, 16). Hydrolysis of GTP or related nucleotides may be involved in microtubule polymerization (17). Although there is no known nucleotide requirement for microtubule depolymerization, ATPases such as the Ca^{++} transport ATPase found in the sarcoplasmic reticulum may play an indirect role in mitosis by regulating or creating the physiological conditions that promote selective depolymerization of microtubules near the spindle poles (8).

We consider it unlikely that vanadate affects chromosome movement by directly altering rates of microtubule polymerization or depolymerization in the mitotic spindle. The level of spindle birefringence and spindle lability to calcium after lysis is unaffected by vanadate, and in vitro microtubule polymerization proceeds at normal rates

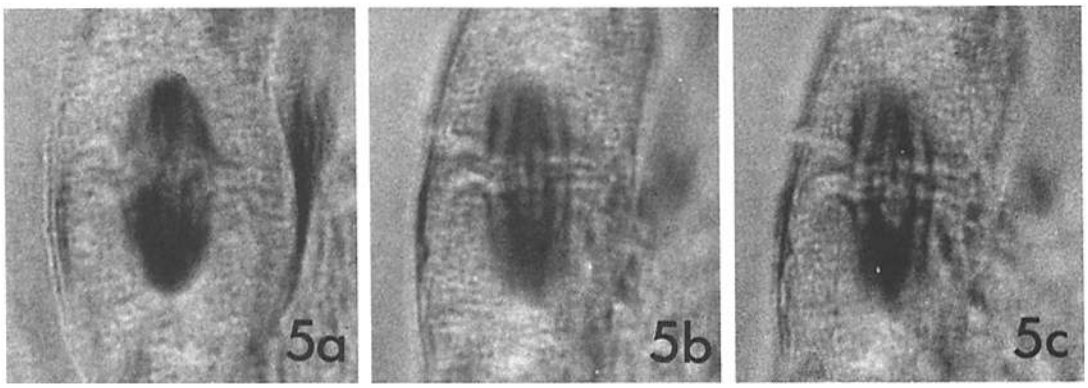

FIGURE 5 *A–C*. Anaphase PtK_1 cell lysed in 100 μM vanadate as seen with polarization optics. Spindle becomes more barrel shaped after lysis. Micrographs are taken at the same compensator settings. *a*, before lysis; *b*, after 5 min in vanadate; *c*, after 11 min in vanadate. × 2200.

even in the presence of 1 mM vanadate. Microtubules assembled in vitro are not stabilized against calcium- or low-temperature induced depolymerization by vanadate.

It has been suggested that calcium fluxes regulate selective depolymerization of kinetochore microtubules and allow chromosomes to approach the spindle poles (8). Well described calcium ATPases such as that found in the sarcoplasmic reticulum are insensitive to vanadate (3). The membrane bound Ca^{++} ATPase that accumulates in the spindles of dividing sea urchin eggs (13) is also insensitive to 100 μM vanadate (C. Petzelt, Heidelberg, personal communication). Inhibition of calcium transport systems should have little effect on spindle elongation. However, we observe that spindle elongation and poleward movement of chromosomes share a similar sensitivity to vanadate. Cells lysed late in anaphase after chromosome-to-pole movements have ceased are also vanadate sensitive.

We suggest that vanadate blocks chromosome movement during mitosis by reversibly inhibiting dynein ATPase activity in the spindle of the lysed cell. This interpretation of our results is consistent with several recent observations. Cross bridges between microtubules are found in many different spindles, although the number of cross bridges is quite small and their biochemical identity is unknown (reviewed in references 14, 16). Dynein has been localized in sea urchin spindles by immunocytological and biochemical means, but it cannot be ruled out that these proteins are cytoplasmic contamination from ciliary dynein pools (15, 20). Sakai et al. (19) have reported that the slow ATP-dependent spindle elongation observed in isolated sea urchin spindles is inhibited by dynein antisera but not by myosin antisera. We have also reported that dynein extracts will alter rates of chromosome movement in lysed PtK_1 cells under some circumstances (14).

The alteration of the fusiform appearance of the spindle after vanadate treatment requires further comment. Lateral interactions between various classes of spindle microtubules mediated by cross bridges like dynein may be essential for preserving spindle shape (14, 16). If inhibition of dynein ATPase activity leads to less cross bridging between microtubules, destabilization of spindle structure will also occur. The changes observed around the spindle poles are unlikely to be a result of selective microtubule depolymerization, since this effect is observed in both anaphase and metaphase cells, does not lead to decrease in spindle length, and is reversible.

We think it is unlikely that vanadate blocks chromosome movement by inhibiting myosin like ATPases. *Dictyostelium* myosin and rabbit skeletal HMM are insensitive to vanadate at concentrations that inhibit anaphase. Although the vanadate sensitive component of the mitotic spindle is not likely to be myosin, it is possible that actomyosin plays a role in mitosis by acting in cooperation with a dynein-microtubule system to move chromosomes. However, myosin antisera injected into sea urchin eggs blocks cleavage but not mitosis (10), and anaphase in spindle isolates is inhibited by dynein but not myosin antisera (19).

Complete inhibition of chromosome movement requires more vanadate than inhibition of ciliary or flagellar beat. The lysed cell contains many other organelles besides the spindle that may bind

vanadate or retard its movement into the interior of the cell. The difference in vanadate sensitivity may also reflect fundamental physiological differences between the two mechanochemical systems. Spindle dynein may be less sensitive to vanadate. Alternatively, it may be a reflection of the different organization of the two organelles. The dynein arms in the cilium are numerous, highly ordered, and interact many times with neighboring microtubules during ciliary movements. The cross bridges in the spindle are few and scattered throughout the spindle (14, 16). It is not known how often they may break and reform during mitosis. Inhibition of ciliary beat by vanadate may occur after only a few dynein cross bridges are inactivated whereas inhibition of anaphase may require that most dyneins in the spindle are inactive.

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