ELECTRON MICROSCOPY OF MITOSIS IN AMEBAE

III. Cold and Urea Treatments: A Basis for Tests of Direct Effects of Mitotic Inhibitors on Microtubule Formation

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ABSTRACT

The mitotic apparatus (MA) of the giant ameba, *Chaos carolinensis*, has characteristic sequences of microtubule arrays and deployment of nuclear envelope fragments. If mitotic organisms are subjected to 2°C for 5 min, the MA microtubules are completely degraded, and the envelope fragments are released from the chromosomes which remain condensed but lose their metaphase-plate orientation. On warming, microtubules reform but show partial loss of their parallel alignment; displacement of the envelope fragments persists or is increased by microtubule reformation. This study demonstrates that cooling causes destruction of microtubules and intermicrotubular cross-bonds and further shows that such controlled dissolution and reformation can provide an in vivo test sequence for studies on the effects of inhibitor-compounds on microtubule subunit aggregation. Urea, at the comparatively low concentration of 0.8 M, inhibited reformation following cooling and rewarming but was ineffective in altering microtubules that had formed before treatment.

INTRODUCTION

The fibrous structure of the mitotic apparatus (MA) is highly labile and is currently thought to be involved directly in the production of most chromosome movements. Physical treatments, such as cooling cells to about 2°C (8, 9) or subjecting cells to pressure of 2000–6000 psi (10, 12, 13), are known to disrupt this organization and also to inhibit chromosome movement.

Mitotic-inhibitor compounds now comprise a long list and have been much studied. One of the major means of inhibition probably involves action of the compound on the microtubules that are a major component of the achromatic portion of the MA. Thus, important studies in this area should deal with the methods by which the action of compounds can be more carefully specified in relation to the fine structural components known to be present.

Several manifestations of inhibitory action can presently be cited, and the possibility that several or all of them may be valid for a single compound has been demonstrated. First, inhibition of fiber formation in prometaphase has long been known, e.g. by colchicine at quite low concentrations (4) and by

vincristine sulfate (6). Second, colchicine at higher concentrations (4) and podophyllin (7) are known to destroy fibers formed before treatment; other compounds may have a similar but more subtle, less than total effect. Third, movement inhibition may be caused by compounds that stabilize the fibrous elements excessively until they are essentially "frozen" or "fixed" but that do not necessarily cause a change in microtubule structure; inhibition of movement in cells where the MA is morphologically unchanged has been reported by Cowdry (2) and Marsland (11). It is important to remember that movement often does not take place even when the fibrous structure is present in the MA although it is also true that most normal chromosome movements do not take place in the absence of the fibrous component. Fourth, inhibition may be the result of an effect on a second structural component whose presence has been postulated (1, 5) but not observed, except for the microtubule-associated material first seen on MA microtubules in giant amebae (17). These four phenomena must ultimately be thought of as resulting from effects on the chemical bonds in microtubules, on the formation-destruction equilibrium of MA microtubules (8, 16),[1,2] and on the specific steps of formation. However, such precision of understanding is far beyond our present knowledge.

The study reported here takes a step toward the development of methods for gaining such specific data on mode of inhibitor action. This work extends the cooling studies involving polarizing microscopy to show reduction and loss of birefringence (9),[2] demonstrates that this effect is actually destruction of microtubules and intermicrotubular cross-bonds, and continues further to show how such controlled dissolution and reformation could provide an in vivo test situation for inhibitor effects on microtubule, subunit aggregation. The major concepts included here were briefly presented earlier (15).

MATERIAL AND METHODS

The giant ameba, *Chaos (Pelomyxa) carolinensis*, was cultured and selected in division stages as previously reported (17). Cold treatment followed the experimental design presented in Fig. 1; cells were fixed at the points designated A–F, some of which constituted controls while others were expected to yield degrading, degraded, reforming, and reformed microtubules in the MA. Variations of the time sequence were also used according to the following ranges: the time B–C (BC) was varied from 5 to 8 min, CD and CE were either 2 or 4 min, EF was 2 or 7 min, and CF was also accomplished in an 18 min, straight-line manner. In all, 17 experiments, including their appropriate controls, were performed with at least 10 experimental organisms used in each; many of the synchronous nuclei of most organisms were examined, after embedding, with the phase-contrast microscope by the quick method described earlier (17), and a few dozen organisms were examined by thin-sectioning and electron microscopy.

Fixation was performed in two ways: first, 1% osmium tetroxide was used with 10^{-3} M $CaCl_2$ at pH 7.6 (sodium acetate and sodium barbital both at 0.014 M) for 20 min, or, second, 2.5% glutaraldehyde similarly buffered (10 min), followed by a short, buffered rinse, and then similarly buffered 1% osmium tetroxide (20 min). The fixation temperature was dictated by the experimental design, but it should be pointed out that this study strongly indicates the inadvisability of fixing dividing cells at 0–4°C for descriptive studies, especially if the tissue is cooled before penetration of the fixative has occurred.

Urea treatments were carried out by using an 0.8 M solution, a concentration found to be relatively nontoxic to living organisms for short exposures. Organisms were placed in urea solution at point B on the design indicated in Fig. 1 and then were carried along the curve CF, with EF as 2 min. Controls included both organisms treated with the urea solution but without cooling and others treated with cooling but no urea.

Dehydration and embedding were identical with the procedures used in previous studies, (17) except that divinyl benzene was added to stabilize methacrylate during electron bombardment. Other embedding media were used but they impaired the phase microscopy and were not adopted routinely in this study. Sections were cut on LKB or Reichert microtomes, stained with potassium permanganate, uranyl acetate, or lead acetate, and examined in an RCA EMU 3F electron microscope at 50 or 100 kv.

RESULTS

Much of the nuclear and MA morphology of this species has been previously described (17). Supplemental information on the nuclear envelope is given here in order to provide a basis for comparison with the effects of cold treatment.

[1] Goode, M. D., and L. E. Roth. Data in preparation.
[2] Kitching, J. A. Unpublished observations.

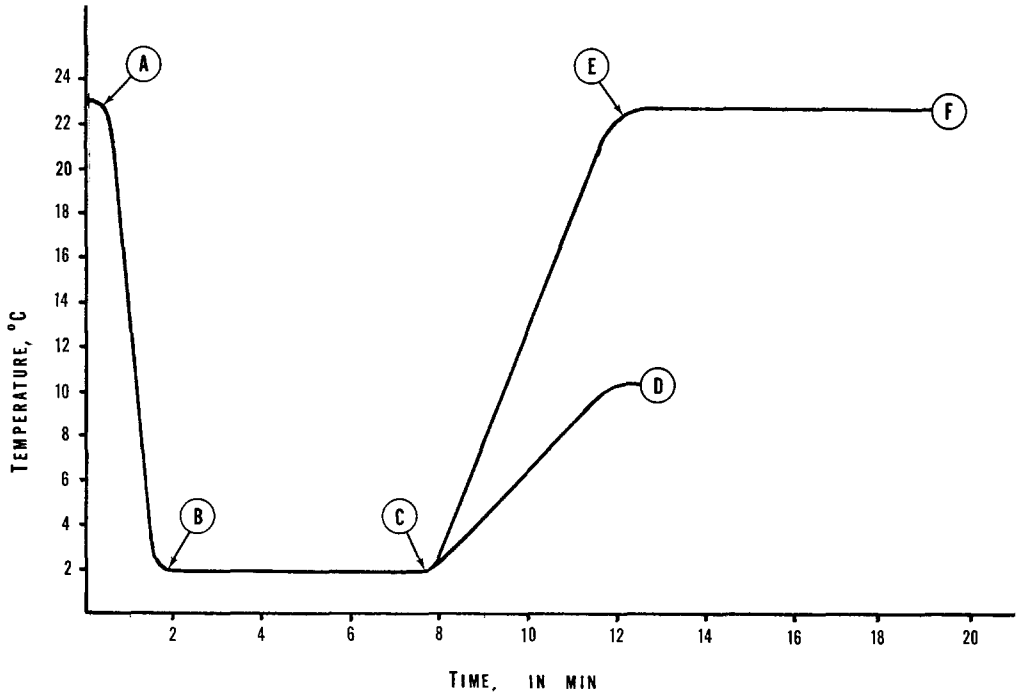


FIGURE 1 Generalized experimental design for the cooling treatments performed. Microtubule destruction is complete at point *C*; test compounds were usually added at *B*. The period of reconstitution of microtubules was varied considerably; at least partial recovery was effected by the *CD* treatment, and typical reconstitution timing is shown by *CF*.

The Nuclear Envelope and Its Fragments During Mitosis

As prophase approaches, nuclei swell to spherical shapes. By the time of chromosome alignment and microtubule formation, the envelope shows frequent interruptions that are fractions of a micron in size. The spherical diameter described by the envelope is slightly larger than the diameter of the chromosome plate, and the envelope fragments are thus several micra from the ends of the MA microtubules.

By metaphase, fragments of the envelope are present in the MA and also as nearly closed vesicles located at the periphery of the chromosome plate (Fig. 2, *E*); pores with annuli are retained in these fragments. Their arrival within the MA occurs in metaphase, whereas the microtubular component that is present at the end of metaphase appears to have been formed largely in prometaphase. Although this movement of membranous elements into the microtubular array is difficult to explain, such a phenomenon has been regularly observed in many mitotic figures from numerous, different amebae.

By early anaphase, fragments (Figs. 3 and 4, *E*) are closer to the chromosome plate (Fig. 3, *C*), are still parallel to the microtubules (Fig. 3, *M*), and still show annuli when frontally sectioned (Fig. 3, *A*). Vesicles that are known to be of pinocytic orgin because they may still show the plasmalemma "fringe" are also present in the MA (Fig. 3, *V*).

In late anaphase, fragments are aligned parallel to the chromosome plate which is completely enclosed except for a few interruptions through which microtubule bundles pass. The early telophase nucleus is a flat disc closely surrounded by an evelope otherwise indistinguishable from the interphase envelope.

Characteristics of the Mitotic Apparatus

The giant ameba typically has an MA that is nonconvergent; all microtubules are largely parallel to each other (Figs. 2 and 3, *M*). By very late anaphase, a partial convergence is seen, and all

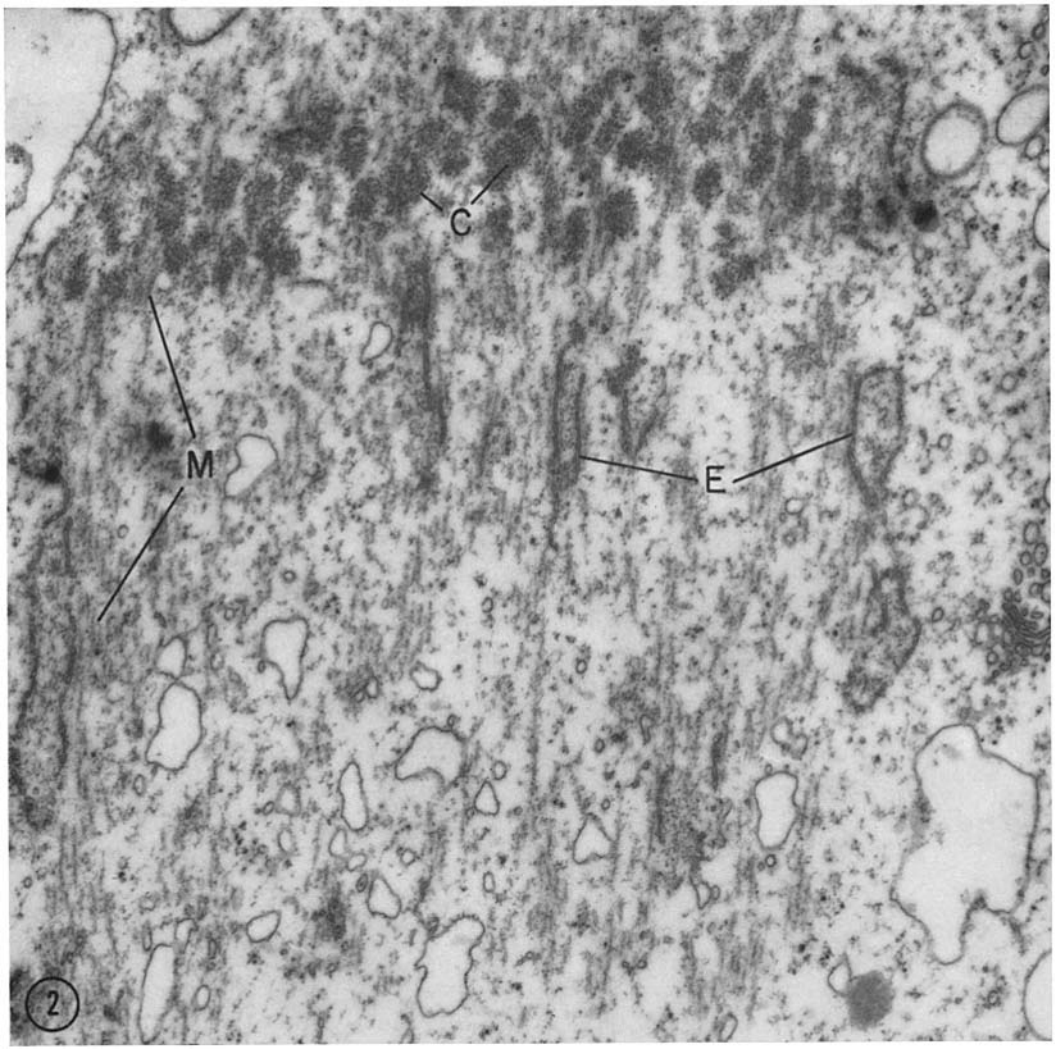


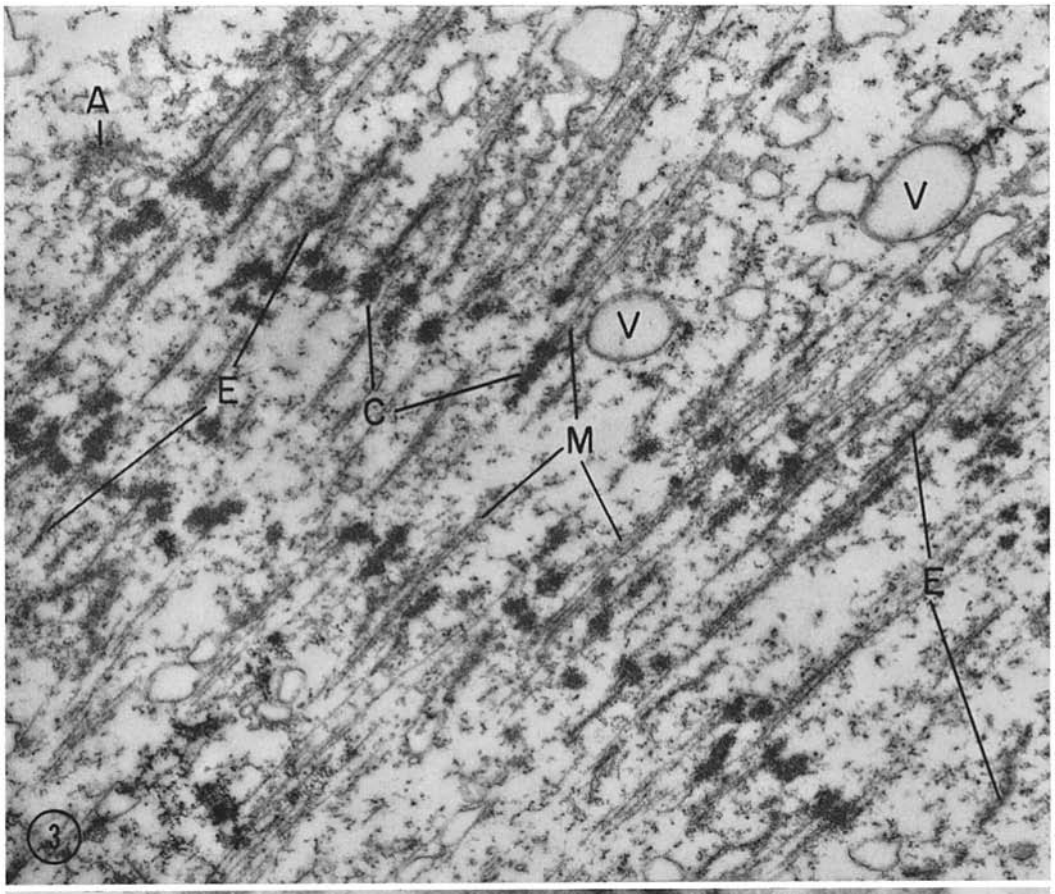
FIGURE 2 Metaphase MA in an ameba without cooling. Fragments of the nuclear envelope (*E*) have moved from their earlier poleward positions and are now parallel to and between microtubules (*M*) in the MA. A concentration of fragments is typically seen at the periphery of the chromosome (*C*) plate and appears as a loop preceding the chromosomes. × 19,000.

microtubules seem to project toward a point that is several micra from their ends.

Chromosomes, which number a few hundred and are all small in size, are usually rather closely packed in a plate that is only three or four times thicker than their own diameter. The plate is flat and planar until the microtubule convergence in late anaphase causes a slight curvature.

Effects of Cold Treatment

The addition of cold (2°C) fixative to organisms held at that temperature for 3 min (3 min past point *B*. Fig. 1) followed by the standard preparative procedures results in a great reduction of microtubular material (Fig. 5, *E*), a slight disarrangement of chromosomes (Fig. 5, *C*), and normally aligned envelope fragments (Fig. 5, *E*).



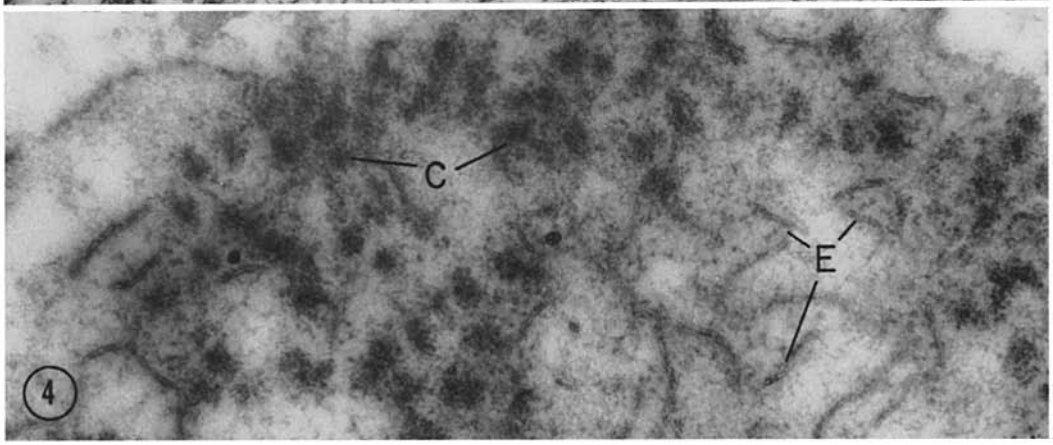


FIGURE 3 Anaphase in an ameba without cooling. Fragments of the envelope (*E*) that still contain annuli (*A*) are present near the chromosomes (*C*) throughout anaphase. By very late anaphase, the fragments will be parallel to the chromosome plate. A few vesicles (*V*) are often included in the MA. *M*, microtubules. × 17,000.

FIGURE 4 Poleward view in anaphase showing frequency of envelope fragments (*E*) and their proximity to chromosomes (*C*). × 29,000.

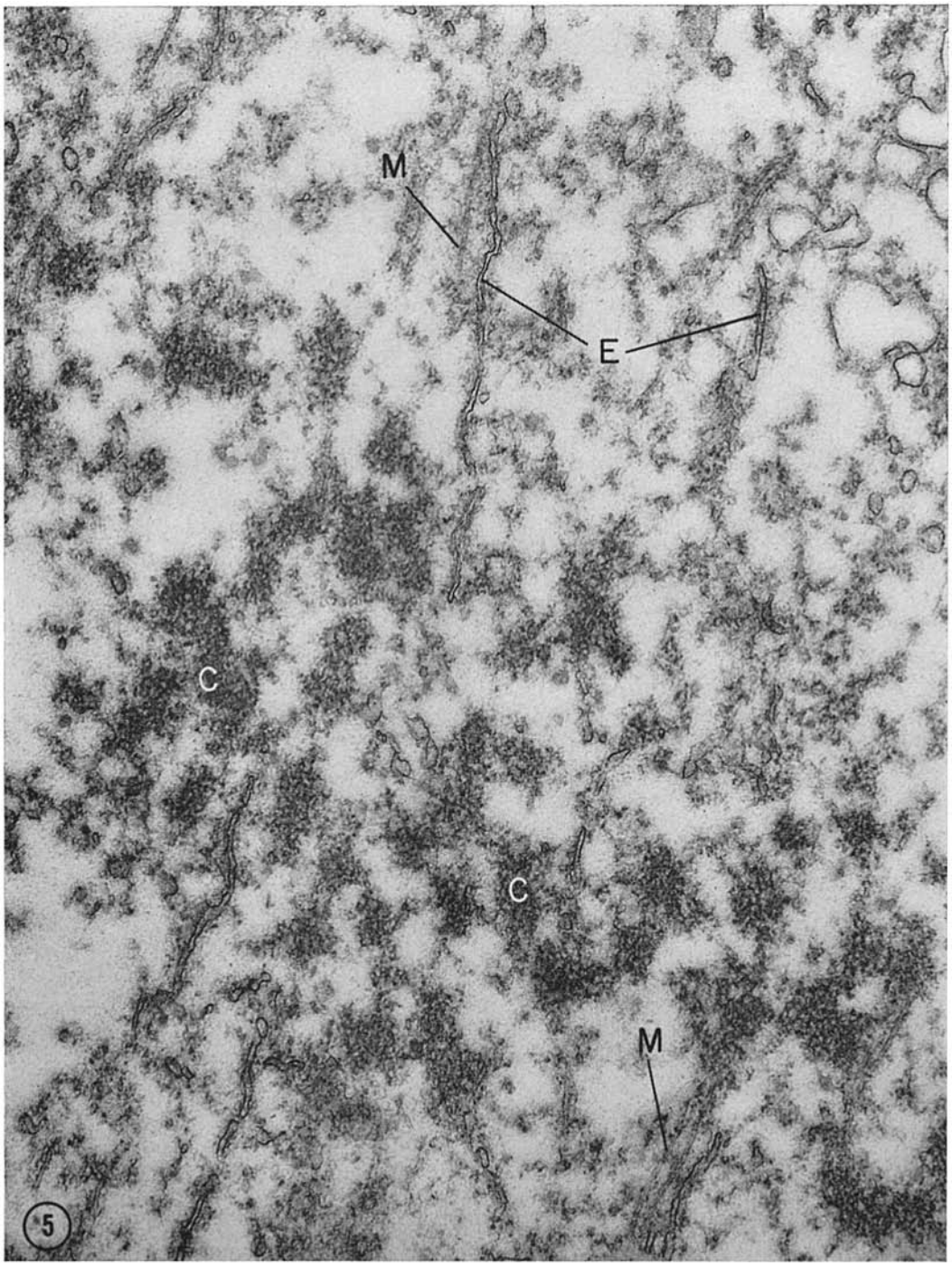


FIGURE 5 Mitotic apparatus from an organism fixed at B + 3 min (See Fig. 1). Chromosomes (*C*) remain condensed and are slightly dispersed, and the number of microtubules (*M*) is greatly reduced. Those microtubules remaining are usually close to the surfaces of envelope fragments (*E*). × 52,000.

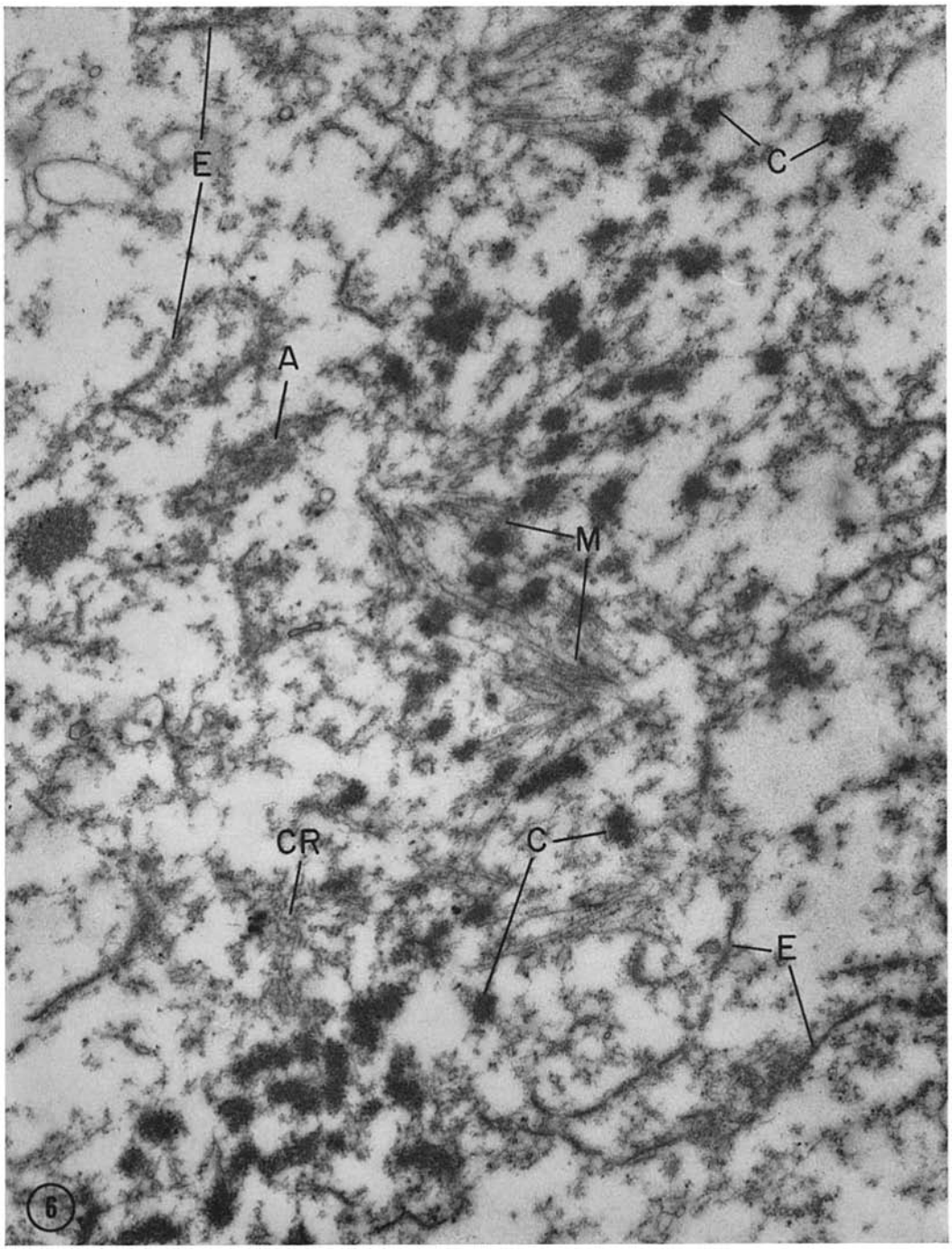


FIGURE 6 Metaphase in an organism fixed at *E* (see Fig. 1) and showing partial reconstitution of the MA. Microtubules (*M*) are about 1 μ long, are poorly oriented, and are attached to chromosomes (*C*); in addition to a tendency to converge, some microtubules are perpendicular (*CR*) to others. Fragments of the envelope (*E*) are widely dispersed but always show annuli (*A*) when frontally sectioned. × 18,000.

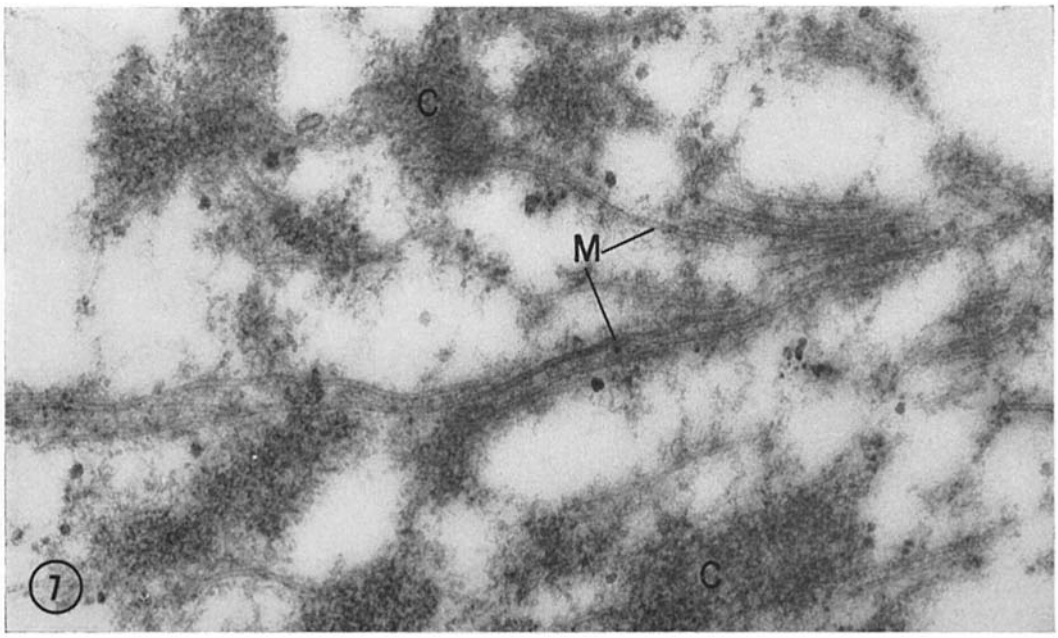


FIGURE 7 Metaphase in an organism where *BC* = 8 min, CE = 2 min, and EF = 1 min. Partial recovery from cold results in condensed chromosomes (*C*) and converging microtubules (*M*) that would not normally be so closely grouped until telophase. × 57,000.

Prolonged exposure to this temperature allowed the envelope fragments to become displaced many micra and to be in considerable disarray. After 5–6 min at 2°C, no microtubules could be seen. Thus, point *C* on Fig. 1 was established as the time of complete microtubule destruction.

Warming the organisms after point *C* causes microtubule reformation. One organism, whose treatment was apparently initiated in metaphase and was similar to that indicated in Fig. 1 except that *C–E* and *E–F* were both reduced to 1 min, showed partial recovery. Chromosomes are scattered, and the envelope fragments (still with annuli, Fig. 6, *A*) are noticeably disarrayed (Fig. 6, *E*). Microtubules are present in large numbers but are short and near chromosomes which thus are implicated in microtubule formation. Parallel arrangement of microtubules is markedly disturbed; cross-sectioned bundles (Fig. 6, *C*) are only a few micra from other bundles more longitudinally sectioned (Fig. 6, *M*). A tendency for localized convergence of microtubules is clearly seen to the extent that, at higher magnification, their distal ends are almost touching each other (Fig. 7, *M*). With increases of the time CF, convergence is reduced.

An organism, whose treatment was probably initiated in early anaphase and utilized the schedule BC = 3, CE = 2, and EF = 3 min shows mitotic figures, similarly affected. Chromosomes, although mostly in a tight plate, show some disarray so that a few lagging chromosomes are seen (Fig. 8, *L*). Continuous microtubules (above the chromosome plate, Fig. 8) are present as well as chromosomal ones, although the latter are about one-half (2.5 μ) the usual anaphase length. The envelope fragments are displaced and have formed what appears to be a double hemisphere (Fig. 8, *E*) which is oriented poleward around each chromosome plate (Fig. 8, *C*). Envelope fragments are characteristically excluded from newly reformed microtubule bundles.

Urea Treatment

After treatment of dividing organisms with 0.8 M urea solutions at room temperature the microtubules appear normal. After similar urea treatment on the schedule BC = 8, CF = 4 min, and

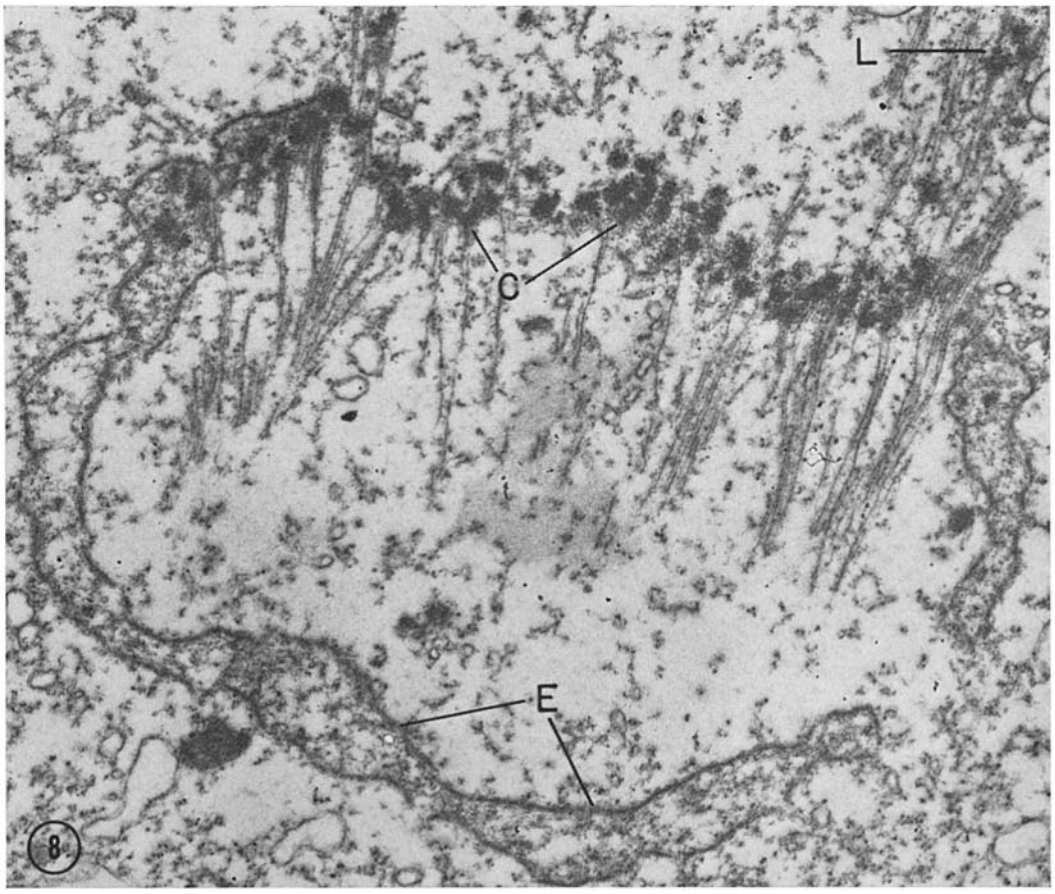


FIGURE 8 Anaphase organism fixed following BC = 3 min and CF = 6 min. Chromosome (*C*) alignment is re-established except for a few lagging chromosomes (*L*). The reforming microtubules have pushed the envelope fragments (*E*) poleward. × 15,000.

urea addition at B + 3 min, microtubules are absent and both chromosomes and envelope fragments are dispersed (Fig. 9.). The control organisms for this experiment were handled on the same schedule of cooling and are typified by the MA in Fig. 6 which is from such a control organism.

DISCUSSION

The Effects of Cold Treatment on the Mitotic Apparatus

Metaphase and anaphase events in the giant ameba are characterized by two phenomena: the sequence of microtubule formation that has been previously described (17) and the sequence of movements of nuclear envelope fragments described in this study. After the breakdown of the nuclear envelope, fragments are found at poleward locations, from which they move into the MA parallel to the microtubular bundles; they move to positions closely apposed and parallel to the chromosome plate by late anaphase. In no case do they appear to be attached to the ends of microtubules. The mechanism for these movements is unknown, but the sequence has been observed repeatedly and must be considered characteristic of mitosis in these cells.

The effects of cold treatment on the MA must similarly be considered as two-fold: the microtubular material is destroyed beyond any recognition and the fragments of the nuclear envelope are released so that they move freely in the cell. As a

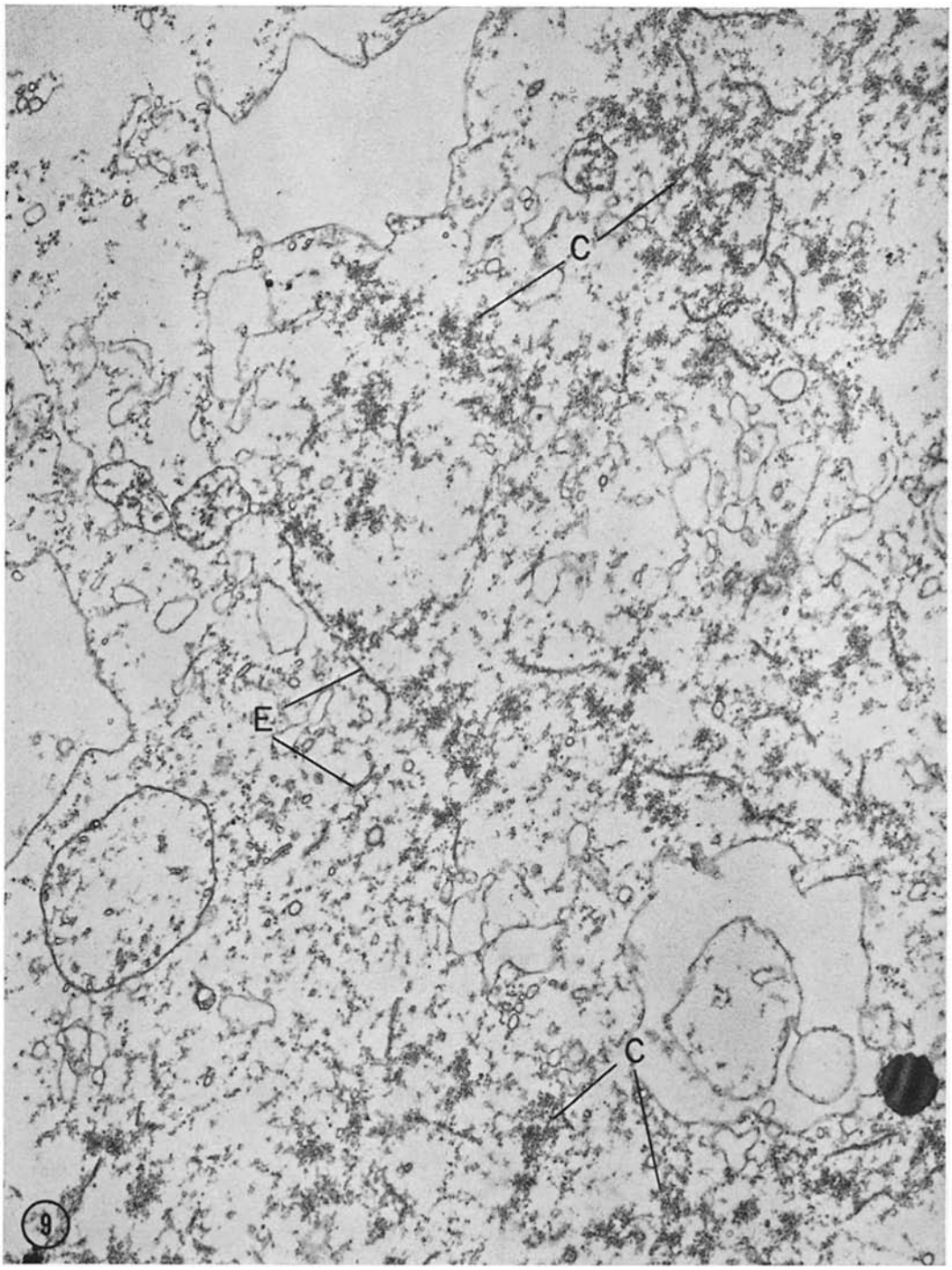


FIGURE 9 An organism fixed at E following addition of 0.8 M urea at B (see Fig. 1). Chromosomes (*C*) are no longer aligned and fragments of the nuclear envelope (*E*) are widely dispersed. No microtubules can be found; similar amebae fixed with urea for the same time but without cooling show normal MA. × 16,000.

result, the chromosomes can and do disperse, the degree of dispersement being determined by the time allowed in the cold; no immediate decondensation of the chromosomes themselves has been observed. Recovery from cold treatment includes the reformation of microtubular material, beginning at the chromosomes, and a resulting displacement of envelope fragments away form the chromosomes; nuclear membrane material is pushed poleward or lost by displacement into the cytoplasm. In addition, the microtubular material is less well aligned, so that several, small groupings, each with pointed, poleward ends, are present; this configuration has not been observed in untreated cells, except in the MA as a whole in late anaphase. That this convergence occurs without the influence of centrioles is also worthy of note.

Intramicrotubular Chemical Bonds

The breakdown of microtubules by cold treatment should be contrasted to the degradation due to lack of calcium in the fixation medium. In the case of cold treatment, the breakdown of microtubules is so complete that no remnants or indications can be observed. However, fixation of these cells in alkaline osmium-tetroxide solutions that are deficient in calcium (lower than 10^{-4} M) allows the microtubules to break down into fine linear elements that are still visible and lie parallel to the original microtubule (17). Thus, cold treatment represents a more complete destruction, probably into individual subunits, whereas fixation involving calcium deficiency may represent breakdown only to polymer chains. The hypothesis follows that the function of calcium in the MA microtubules may have to do with the lateral cross-bonding of linear chains.

Urea, which impairs normal hydrogen bonding and weakens salt linkages, interfered with microtubule reformation under the conditions used in this study so that no reaggregation of even a partial nature occurred. Such a finding is in agreement with currently held concepts that both hydrogen bonds and salt linkages are important in movement systems. Since it is likely that both of these bondings are involved in microtubule formation, the possibility exists that mitotic-inhibiting compounds may be found which inhibit one but not both bonds and allow reformation only of chains that have a width equal to the monomer diameter. In this regard, cells are known (16) that have fibrous components of two types, one composed of microtubules and the other of much finer linear elements; it is likely that naturally occurring conditions or protein differences allow only one of two possible bondings to occur.

The Gel Nature of the Mitotic Apparatus

Although much study has been directed toward the microtubular components of the MA, little attention has been paid to their interrelationships with other components of the cell. The observation that material adheres to microtubule surfaces has been directed to two hypotheses: (*a*) this material is the same as that composing the microtubules and has been, or will be, incorporated into the microtubules (16, 18); (*b*) it is different material, perhaps that suggested for a two-component movement system (1, 5). However, attention should also be directed, on the basis of this study, to the role of this material in holding the MA together and connecting it to adjacent structures. Release of nuclear envelope fragments results from cold treatment and microtubular destruction. Nuclear envelope fragments are free to disperse into the cytoplasm in cold-treated cells even though no direct contact of microtubule and membrane surface has been observed or even seems possible from the spatial relationships normally observed.

These observations are reminiscent of the classical concept of the gel nature of the MA. This concept is an old one and can be substantiated from many studies that utilized isolation, micromanipulation, and centrifugation; Mazia (14) summarizes the data well and concludes that the MA must be considered as both "a region and a body." However, the concept is extended by this study to include interrelationships with such structures as nuclear envelope fragments and perhaps to others such as mitochondria, which are integrally related to the MA in another ameba (3), and Golgi bodies, which may be precisely positioned in relation to the MA (18). In these cases, the gelation seems to include structures peripheral and adjacent to the MA and probably aids in the equal distribution of some organelles to the daughter cells.

The Testing of Mitotic Inhibitors and Poisons

This study has established that the absence of birefringence in cold-treated, mitotic amebae is actually destruction of the microtubular com-

ponent. This controlled degradation and its subsequent reversal allows a test situation for experimenting with only those stages in microtubule formation that are involved with the aggregation of subunits; steps in the synthesis of subunits should be largely excluded by the time parameters involved.

Considerable importance should be placed on the ability to determine the particular events in the division process that are affected by a given inhibiting substance. The events in microtubule formation can probably be designated as synthesis, subgroup aggregation into chains, chain aggregation into microtubules, and microtubule-array formation (16); the events in chromosome movement are thought to be microtubule growth and diminution (9, 16). To be able to attribute the action of a given compound to particular steps would be a major advance in the understanding of mitotic inhibition and control. Although the technique proposed here cannot yet distinguish between the last steps cited, it is possible to separate the synthesis from the aggregations that are involved, and thus a step is taken in the desired direction. Although similar, specific information on colchicine action is being obtained by the elegant work of Taylor (19), the method proposed here has primary merit as a rapid screening method that allows quick testing of numerous compounds on numerous cells.

Two test sequences are possible and would determine whether a compound tends to "freeze" or to impair formation of the MA microtubules. First, if a compound is added to the cellular environment immediately before cooling, time for its penetration into the cell allowed, and the temperature elevated in the intracellular presence of the test compound, it will be possible to observe whether microtubular reconstitution or aggregation has been inhibited. This sequence tests for disruptive effects of a compound. Such a test has been successfully applied in this study; dilute solutions of urea have been shown to impair the reformation of microtubules while the same concentration does not affect already formed microtubules treated at the usual culture temperatures. Second, this cooling sequence can also be extended to test for compounds that may stabilize the MA microtubules excessively. In this case, the compounds would be added at or before *A* (Fig. 1), and the delay in or lack of disintegration would be observed.

Similar tests have been and will be carried out on clinically significant compounds. Although tests with colchicine, mustine, and aminopterin have yielded negative or questionable results, no demonstrations of the intracellular presence of the compound during reconstitution have yet been possible; further studies will ascertain whether penetration has been achieved by the time of reconstitution. Similarly, the slowness of testing by electron microscopy must be improved. Since this study has shown that the loss of birefringence observed in the light microscope during cold treatment is actually microtubule destruction, the procedure can be transposed to a light microscope, assay method, at least for screening purposes.

An alternate approach in the test sequence would be microtubule destruction by high pressure (10, 12, 13). Although pressure effects are known to stop chromosome movement and cause disappearance of fibrils in the MA, the destruction of MA microtubules can only be inferred; however, the studies of microtubular destruction in *Actinosphaerium* by pressure, cold, and colchicine (20)[2,3] point convincingly in that direction. Increased pressure, compared to reduced temperature, might hasten penetration of the test compound into the cell. Precise information on the specific mode of action of biologically significant compounds on microtubule formation needs to be collected and will inform us about both the formation process under normal conditions and the impairment of that process under pathological conditions.

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