THE MITOTIC APPARATUS

Structural Changes after Isolation

R. E. KANE, Ph.D., and ARTHUR FORER, Ph.D.

From the Department of Cytology, Dartmouth Medical School, Hanover, New Hampshire, and the Marine Biological Laboratory, Woods Hole, Massachusetts

ABSTRACT

The fibrous structure of the mitotic apparatus (MA) isolated from dividing sea urchin eggs undergoes no changes visible in phase contrast during extended storage, but the solubility of the MA rapidly decreases after isolation. Polarization microscopy shows that a decrease in the birefringence of the MA also occurs after isolation and is correlated with the loss of solubility. This loss of birefringence indicates that some structural change takes place during this period, and such a change was demonstrated by means of electron microscopy. The tubular filaments which form the spindle of the intracellular MA and of the freshly isolated MA were found to break down during storage to rows of dense granules, this loss of continuity presumably accounting for the loss of birefringence. The interrelations of the observed changes and the significance of these observations for investigations on the isolated MA are discussed.

INTRODUCTION

Recent investigations on the stabilization of the mitotic apparatus (MA) have led to the development of isolation methods which cause no apparent structural or chemical change in the MA. Following the direct isolation of the MA from dividing sea urchin eggs by Mazia *et al.* (18) using a disulfide containing glycol, Kane (13) demonstrated that the MA could be stabilized by a simple long chain glycol and that the presence of disulfide groups was not required for isolation. A 1 M solution of hexanediol, buffered at a slightly acid pH, was sufficient to stabilize the mitotic apparatus and was also effective in liberating the MA from the cell by osmotic lysis. Further investigations on the mechanism of stabilization (15) have shown that a variety of organic solvents can be substituted for the glycol and that the concentration of solvent required and the pH of the medium are related in a manner which suggests that a conventional protein solubility curve is involved.

The ultimate proof of the physiological condition of the isolated mitotic apparatus would obviously be the demonstration of functional activity *in vitro*. But recent theories of the mechanism of chromosome movement, which postulate that changes in the relative amounts of oriented and unoriented material are involved in the shortening of the fibers (9), suggest that *in vitro* activity of the MA may require the presence of additional cytoplasmic components which may not be retained during isolation. In the interim the condition of the isolated MA must be evaluated by other methods.

The effects of isolation on the fine structure of the mitotic apparatus have been investigated by means of electron microscopy (14). The spindle and asters of the isolated MA were found to be composed of tubular filaments, similar to those seen in the dividing sea urchin egg (3, 4) and in other dividing cells (1, 20, 21). The isolation proc-

ess thus has no apparent effect on the basic fibrous structure of the MA.

Another criterion that has been used in evaluating the condition of the isolated mitotic apparatus is its ease of solubility. Since the intracellular MA is a very labile structure, one expects the isolated MA to be very easily soluble. One of the major objections to the original isolation method of Mazia and Dan (17) was that the resulting MA were difficult to dissolve; to overcome this objection, improved methods were developed which yielded more soluble MA (18, 13). Mitotic apparatuses isolated by the glycol-pH method dissolve slowly in water and very rapidly in salt solutions, proving that irreversible stabilization has not occurred during isolation.

However, the solubility properties of the mitotic apparatus change rapidly after isolation. Zimmerman (26) noted that MA isolated by the original cold ethanol procedure became less soluble with time, and Kane (13) found that the solubility of glycol-isolated MA decreased during storage, even when the MA had been maintained in the isolation medium. These solubility changes make experiments requiring larger quantities of material more difficult, as MA cannot be accumulated over a period of time, but must be used immediately after isolation. In order to prevent or reverse this loss of solubility, more information was needed concerning the nature of the changes occurring in the MA after isolation.

Evidence of other postisolation changes in the mitotic apparatus was obtained by polarization and electron microscopy. Though a considerable number of birefringence studies have been made on dividing marine eggs (6, 7, 10, 19, 22–25), and the mitotic apparatuses isolated in the original experiments of Mazia and Dan were shown to be birefringent (17), this technique has been little used in the study of the isolated MA. In the present experiments, polarization microscopy first provided evidence that a change in the structural organization of the MA accompanied the loss of solubility, and the nature of this change was then determined by electron microscopy.

MATERIALS AND METHODS

These investigations were carried out on mitotic apparatuses isolated from eggs of the sea urchins *Arbacia punctulata* and *Strongylocentrotus purpuratus* at metaphase of the first division. The isolation procedure has been described in detail in previous publications (13, 15). The last step of the procedure used here involves lysis of the cells in a 1 M (12.5 per cent *v/v*) solution of hexylene glycol (2-methyl 2,4-pentanediol) containing 0.01 M KH_2PO_4 adjusted to pH 6.4 with KOH. After lysis the temperature was rapidly reduced to 0°C and the MA spun down and resuspended two times in the same glycol media to reduce cytoplasmic contaminants. They were then stored under the conditions and for the times noted in each experiment.

The solubility of the isolated mitotic apparatus was tested by flowing the desired solution between the coverglass and slide or by mixing double strength solutions with an equal volume of MA suspension. Large volumes of MA were dissolved by centrifuging out of the glycol mixture and adding the appropriate test solution.

Birefringence studies were carried out with an American Optical Super-Bio Polarizing Microscope with rectified optics (8, 11), which was used through the courtesy of the Marine Biological Laboratory, Woods Hole, Massachusetts, and Dr. Shinya Inoué. Spindle retardations were calculated from the angles of the spindle and of the 17-mμ compensator with respect to the crossed polars, at extinction (maximum darkness) of the spindle (5).

Electron microscope studies were carried out on isolated MA fixed for 30 minutes at 0°C in 1 per cent osmium tetroxide in 1 M hexylene glycol containing 0.01 M KH_2PO_4, pH 6.4. After fixation the MA were dehydrated in a graded ethanol series, with 1 per cent phosphortungstic acid included in one change of absolute ethanol. The samples were then embedded in a mixture of 80 per cent butyl and 20 per cent methyl methacrylate containing 1 per cent benzoyl peroxide as catalyst. The capsules were polymerized at 60°C for 24 hours. Sections were cut on an LKB Ultrotome at settings of 200 to 400 A, and picked up on Formvar- and carbon-coated grids. They were examined in a Siemens Elmiskop I at 60 kv, using a 200-μ condenser and a 50-μ objective aperture.

RESULTS

Solubility Changes after Isolation

The stability induced by the isolation medium should be reversible if no chemical changes occur in the mitotic apparatus. Since the stabilizing action of the isolation medium used in these experiments has been shown to depend on the presence of glycol and a slightly acid pH (15), the MA would be expected to dissolve upon a reduction in the glycol concentration and an increase in the pH of the suspending medium. This is done experimentally by transferring the MA from the pH 6.4 1 M glycol solution used for isolation to water

buffered at pH 7.5 with 0.01 M KH_2PO_4. In this solution breakdown of the MA begins first in the spindle region, freeing the chromosomes from the apparatus and causing separation of the asters (13). The asters then dissolve more slowly; small central remnants remain after 24 hours at 4°C. Fine granules, possibly representing the vesicles seen in the MA with the electron microscope (14), are released as the spindle and asters break down.

Although this solubility in water is important in demonstrating the reversibility of stabilization, it is too slow to be used in testing solubility changes during storage. The mitotic apparatus can be dissolved much more rapidly by treatment with solutions of higher ionic strength (13). In 0.6 M KCl the MA dissolves immediately and with no evidence of differential effect, the entire MA collapsing to a mass of fine granules which rapidly disperse. Unlike the dissolution of the MA in water, this reaction is not sensitive to pH nor to the presence of glycol, and the MA will dissolve in 0.6 M KCl in 1 M glycol at pH 6.4. These characteristics are very useful when following changes in solubility, since it is not necessary to wash out the small amount of isolation medium that may be carried over with the mitotic apparatus. This solution has been used in all the solubility experiments reported here.

The solubility of the mitotic apparatus in 0.6 M KCl solution decreases with time after isolation. Instead of the almost instantaneous collapse seen in freshly isolated MA, breakdown becomes slower, with evidence of differential solubility: the spindle region dissolves first, followed by the disappearance of the asters. The time required for the dissolution of the MA increases with longer storage until the MA finally becomes insoluble and shows no response to 0.6 M KCl. These changes take place in a few hours at room temperature, but can be retarded by storage at a lower temperature. The routine isolation procedure involves the transfer of the MA to 0°C immediately after cell lysis, and storage at 0° or 4°C. Under these conditions the MA remains completely soluble for several hours, allowing the accumulation of MA from a number of egg batches; but solubility gradually decreases, and after 24 hours dissolution is slower and often only partial. After 48 hours at 4°C there is no response to salt solution.

Birefringence Changes after Isolation

These changes in the solubility of the mitotic apparatus are not accompanied by changes in the phase contrast image, for MA stored for extended periods (Fig. 1) appear identical with those freshly isolated (13, 15). The first evidence of structural changes in the MA during storage was given by polarization optical studies. Mitotic apparatuses isolated by the glycol-pH method have an average retardation of 4 mμ immediately after isolation, with a range from 3 to 5 mμ. The birefringence of the MA within the living cell is difficult to measure in *Arbacia* and *Strongylocentrotus* eggs because of the yolk granules present, but measurements on the more transparent eggs of the sea urchin *Lytechinus variegatus* have given retardation values in this range and have also shown that marked changes in birefringence occur over a period of a few minutes during metaphase and anaphase (12). Since division synchrony is never perfect in any batch of eggs, these rapid changes in retardation may contribute to the variations in the measured values for the freshly isolated MA.

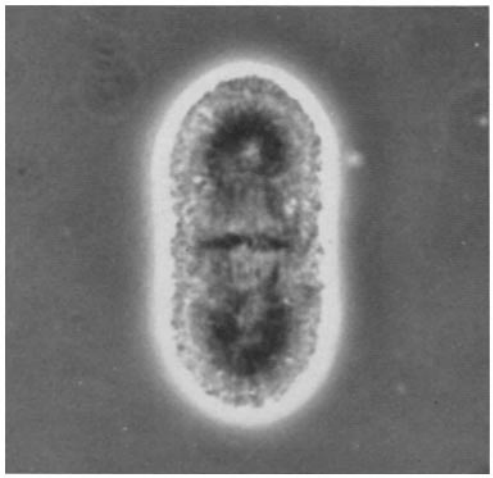

FIGURE 1 Phase contrast photomicrograph of an isolated mitotic apparatus maintained at room temperature for 17 hours. × 1000.

The loss of visible birefringence of the isolated mititoc apparatus at room temperature is illustrated in Fig. 2, and the decrease of the measured retardation values with time for two cases is given in Fig. 3. The upper line of Fig. 3 (open circles) shows the decline of birefringence for a mitotic apparatus that had been isolated and maintained at 0°C for approximately 1 hour before being transferred to room temperature. Storage at 0°C for short periods has a negligible effect on the birefringence, and the MA had a retardation of

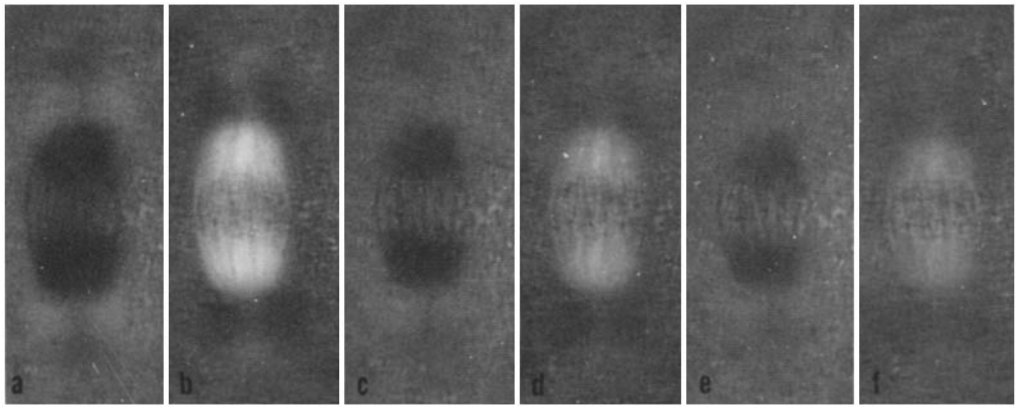

FIGURE 2 Loss of birefringence of the isolated mitotic apparatus at room temperature. A, C, and E were photographed with subtractive compensation, B, D, and F were photographed with additive compensation. All photographs are of the same mitotic apparatus, taken at the following times after transfer to room temperature: A, 13 minutes; B, 15 minutes; C, 45 minutes; D, 47 minutes; E, 65 minutes; F, 67 minutes. × 1000.

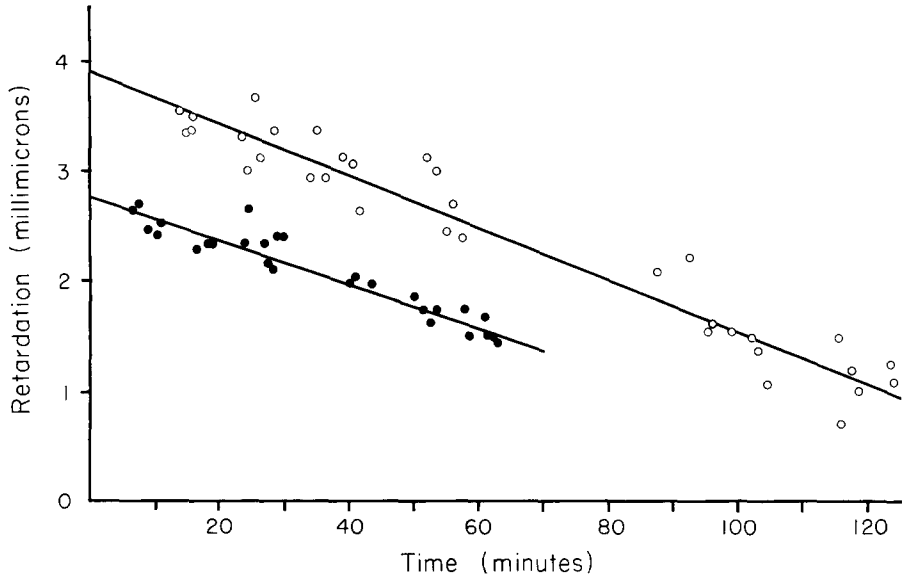

FIGURE 3 Decrease of retardation with time at room temperature. Open circles, mitotic apparatus maintained at 0°C for 1 hour before transfer to room temperature. Closed circles, mitotic apparatus maintained at 0°C for 5 hours before transfer to room temperature.

4 mμ at the beginning of the experiment. The retardation decreased rapidly after transfer to room temperature, falling to approximately 1 mμ in 2 hours. Accurate measurements of retardation cannot be made below this value, but extrapolation indicates that all birefringence would be lost in 3 hours.

Mitotic apparatuses held at 0°C for longer periods before transfer to room temperature have a reduced birefringence, but the remaining retardation is lost at approximately the same rate, as shown by the lower line (closed circles) of Fig. 3. This MA was held at 0°C for 5 hours and when brought to room temperature had less birefrin-

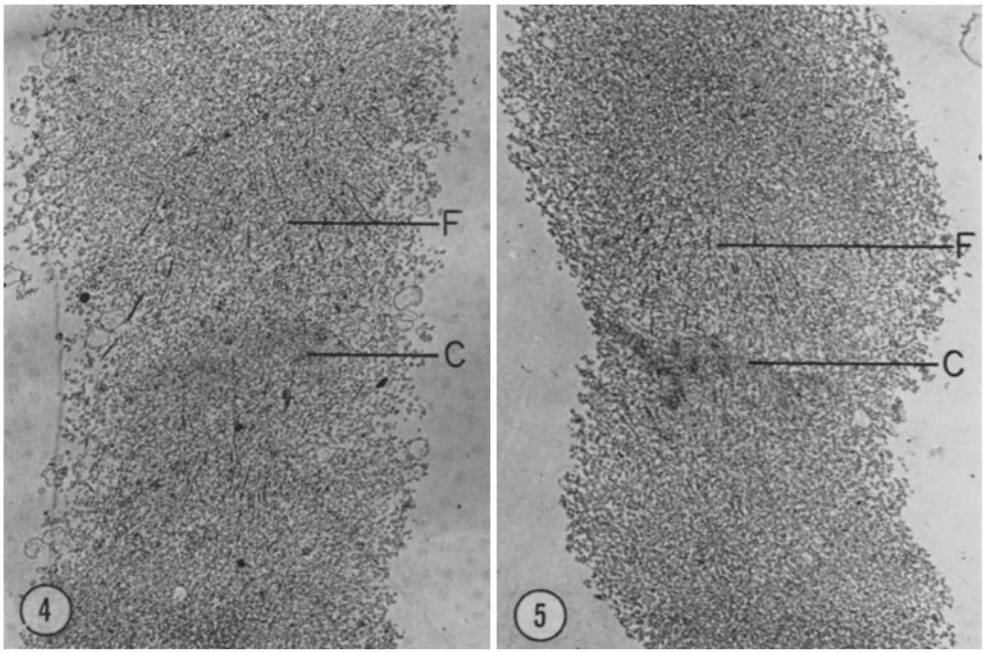

FIGURE 4 Electron micrograph of a freshly isolated mitotic apparatus. *C*, chromosomes; *F*, spindle fibers. × 3700.

FIGURE 5 Electron micrograph of a mitotic apparatus maintained at room temperature for 17 hours. *C*, chromosomes; *F*, spindle fibers. × 3700.

gence than the previous MA, but this birefringence is lost at approximately the same rate as in the first case. Mitotic apparatuses stored for longer periods at 0°C show proportionately greater losses of birefringence. Retardation is much reduced after 24 hours and is completely absent after 48 hours at this temperature. The loss of birefringence at room temperature and at low temperature thus parallels the loss of solubility described above. The birefringence of the MA is retained through fixation and embedding, and the fine structural changes involved were investigated by means of electron microscopy.

Fine Structure Changes after Isolation

The decrease in birefringence of the mitotic apparatus provides evidence that some modification of the oriented structure of the MA occurs after isolation, while the absence of detectable change in the fibrous structure visible in phase contrast indicates that this modification is below the optical limit of resolution. Comparison of very low power electron micrographs of a freshly isolated MA and of one which had been maintained at room temperature for an extended period (Figs. 4 and 5) explains the unchanged phase contrast image. Although the magnification is even greater than that of the phase contrast figure, the superficial structure of the MA appears unchanged. The chromosomes are present in the metaphase configuration, although they are somewhat extracted (4) and less visible at this pH than at pH 6.0 (14). The fibrous structure of the spindle of the stored MA appears identical with that of the freshly isolated MA.

Comparison of such MA at higher magnification demonstrates, however, that fundamental changes have occurrrd in the fine structure of the mitotic apparatus. In the freshly isolated MA (Fig. 6) the spindle region contains tubular filaments approximately 200 A in diameter, which can be seen in favorable sections to extend for long distances through the spindle. In the stored MA (Fig. 7) these filaments appear to have broken down, giving rise to long rows of dense granules having a diameter similar to that of the filaments they replaced. The linear arrangement of these granules accounts for the apparent persistence of the fibrous

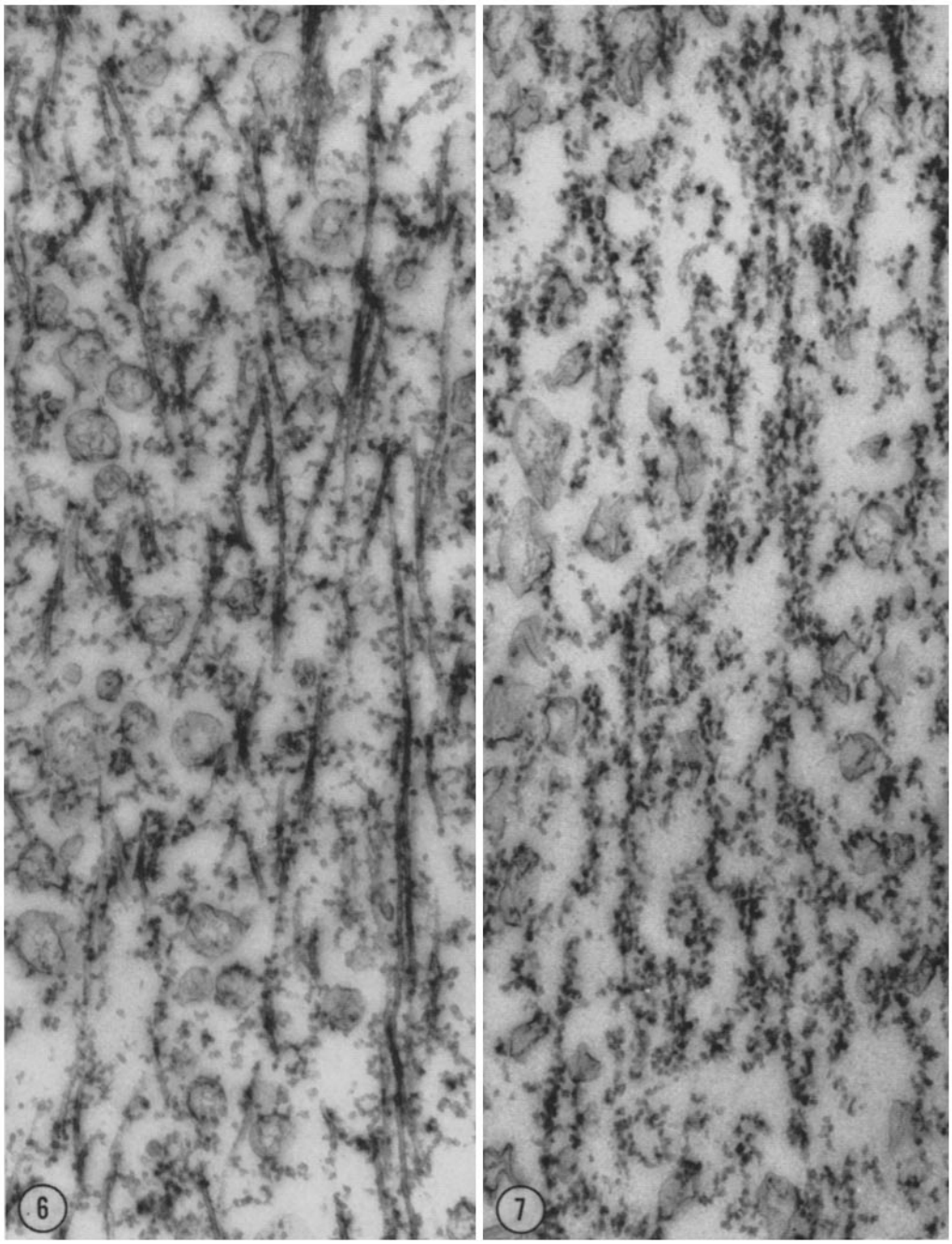

FIGURE 6 Electron micrograph of the spindle region of a freshly isolated mitotic apparatus. × 46,500.
FIGURE 7 Electron micrograph of the spindle region of a mitotic apparatus maintained at room temperature for 17 hours. × 46,500.

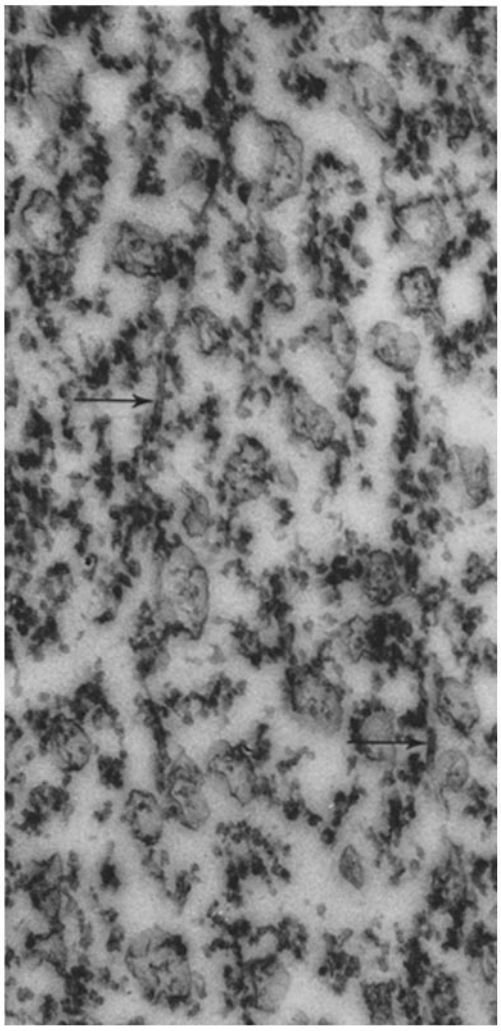

FIGURE 8 Electron micrograph of the spindle region of a mitotic apparatus maintained at room temperature for 3 hours. Remnants of tubular filaments at arrows. × 65,500.

structure in phase contrast, since such a loss of continuity will not be visible with the light microscope. The fact that freshly isolated MA which contain filaments (Fig. 6) are birefringent, whereas stored MA in which the filaments have broken down (Fig. 7) are without birefringence, suggests, however, that the integrity of the filaments is required for birefringence.

This conclusion is strengthened by other experiments which show that the loss of birefringence and of filamentous structure occur in parallel. The loss of birefringence of the MA takes place in approximately 3 hours at room temperature, and electron micrographs show that the loss of continuity of the filaments is almost complete at this time, although a few scattered and discontinuous remnants of tubular structure remain (Fig. 8). Storage at low temperature retards the loss of birefringence and also slows the disappearance of the filaments, but after 48 hours at 0°C no measurable birefringence remains and the loss of filamentous structure is also complete.

DISCUSSION

Of the three postisolation changes observed here—loss of solubility, of birefringence, and of continuity of the filaments—the latter two appear to be directly related. Although regions of optical anisotropy would not necessarily have different densities and be visible in the electron microscope, it would be surprising if ordered anisotropic fine structure seen in the electron microscope did not contribute, at least in part, to the observed birefringence. Since MA fixed immediately after isolation retain their birefringence through embedding, and such preparations have been shown by electron microscopy to contain the filaments, and the loss of birefringence during storage is correlated in time with the breakdown of the spinde filaments, it is reasonable to attribute the birefringence to the presence of the filaments. Studies with the mitotic inhibitor colchicine lend additional support to this conclusion, since the spindle filaments are not present after colchicine treatment (1) and such treatment has also been shown to reduce spindle birefringence (6).

Such a relation between birefringence and the presence of filaments is of particular interest in relation to recent experiments on living cells with an ultraviolet microbeam, which indicate that birefringent chromosome fibers are not necessary for normal anaphase chromosome movement, as chromosomes could move to the pole in anaphase even when there was an area of greatly reduced birefringence on their chromosomal fiber (2). If the birefringence of the spindle in living cells is due to the presence of the 200-A filaments and a reduction in birefringence reflects a loss of these filaments, as would be suggested by the experiments reported here, then the microbeam experiments lead to the conclusion that the continuity of the filaments is not necessary for anaphase chromosome movement. This possibility can be directly tested by electron microscope investigation of the structure of spindles irradiated with an

ultraviolet microbeam in which the chromosomes can continue to move.

The structural change in the mitotic apparatus after isolation occurs in parallel with the loss of solubility, but it is not known at present whether these changes are directly related. From their appearance in the electron micrographs, one might expect the stored MA to be more easily soluble than those freshly isolated, for they appear to be already partially broken down. It is not immediately obvious from the micrographs just what is responsible for maintaining the integrity of the stored MA, for with the loss of the filaments there appears to be no continuous element present. Electron microscope studies of the dissolution of the freshly isolated MA in 0.6 M KCl (16) show that this solution causes the breakdown of the filaments, which is followed by the collapse of the MA. The stored MA, which are without continuous filaments, are as stable mechanically as those freshly isolated and, in addition, are completely resistant to the action of solutions of high ionic strength.

These postisolation changes in the structure and properties of the mitotic apparatus require that experiments on the isolated MA be carried out as rapidly as possible after isolation. Investigations on the soluble components of the MA are obviously possible only during the period in which the MA can be dissolved, but even during this period there is a gradual appearance of differential solubility, and the composition of the soluble fraction may thus change with time after isolation. Study of the structure and function of the filaments must also be limited to the short period during which the filaments persist after isolation. The full utilization of the isolated MA in the study of cell division clearly requires the development of methods for preventing these changes and maintaining the MA in the condition in which it is isolated.

In the present investigation it was shown that these changes can be retarded but not prevented by storage of the MA at low temperature. This is the opposite of the effect observed in the living cell, where exposure to low temperature abolishes the birefringence of the spindle (6). However, the mechanism of the temperature effect in the two cases is quite different: in the living cell the reduction in birefringence is reversible and is believed to be due to the effect of temperature on an equilibrium between oriented and unoriented material (9), whereas in the case of the isolated MA storage at low temperature retards the irreversible loss of birefringence due to the breakdown of the filaments. The reversible temperature effect and related functional activities of the mitotic apparatus will probably not be duplicated *in vitro* until the filamentous component of the MA isolated by present methods is supplemented by additional cytoplasmic components.

This investigation was supported by a Public Health Service Research Career Program award, 1-K3-GM-20229, and a Public Health Service Research Grant, GM-08626, to Dr. Kane, and also by Public Health Service Research Grant CA-04552 and National Science Foundation Grant G-19487 awarded to Dr. Shinya Inoué. Dr. Forer was under tenure of Public Health Service Predoctoral Fellowship GPM-10513.

Received for publication, August 5, 1964.

REFERENCES

1. DALES, S., *Proc. Nat. Acad. Sc.*, 1963, **50**, 268.
2. FORER, A., Ph.D. thesis, Dartmouth College, 1964.
3. HARRIS, P., *J. Biophysic. and Biochem. Cytol.*, 1961, **11**, 419.
4. HARRIS, P., *J. Cell Biol.*, 1962, **14**, 475.
5. HARTSHORNE, N. H., and STUART, A., Crystals and the Polarizing Microscope, London, Edw. Arnold Ltd., 1960.
6. INOUÉ, S., *Exp. Cell Research, Suppl.*, 1952, **2**, 305.
7. INOUÉ, S., *Chromosoma*, 1953, **5**, 487.
8. INOUÉ, S., *in* Encyclopedia of Microscopy, (George Clark, editor), New York, Reinhold Publishing Co., 1961, p. 480.
9. INOUÉ, S., *in* Primitive Motile Systems in Cell Biology, (R. D. Allen and N. Kamiya, editors) New York, Academic Press, Inc., 1964, p. 549.
10. INOUÉ, S., and DAN, K., *J. Morphol.*, 1951, **89**, 423.
11. INOUÉ, S., and HYDE W. L., *J. Biophysic. and Biochem. Cytol.*, 1957, **3**, 831.
12. INOUÉ, S., and SATO, H., personal communication.
13. KANE, R. E., *J. Cell Biol.*, 1962, **12**, 47.
14. KANE, R. E., *J. Cell Biol.*, 1962, **15**, 279.
15. KANE, R. E., *J. Cell Biol.*, 1965, **25**, No. 1, Pt. 2, 137.
16. KANE, R. E., unpublished observations.
17. MAZIA, D., and DAN, K., *Proc. Nat. Acad. Sc.*, 1952, **38**, 826.
18. MAZIA, D., MITCHISON, J. M., MEDINA, H., and

HARRIS, P. J., *J. Biophysic. and Biochem. Cytol.*, 1961, **10**, 467.
19. MONNE, L., *Arkiv. Zool.*, 1944, **35A**, No. 13.
20. ROBBINS, E., and GONATAS, N. K., *J. Cell Biol.*, 1964, **21**, 429.
21. ROTH, L. E., and DANIELS, E. W., *J. Cell Biol.*, 1962, **12**, 57.
22. SCHMIDT, W. J., *Arch. exp. Zellforsch.*, 1937, **19**, 352.
23. SCHMIDT, W. J., *Chromosoma*, 1939, **1**, 253.
24. SWANN, M. M., *J. Exp. Biol.*, 1951, **28**, 417.
25. SWANN, M. M., *J. Exp. Biol.*, 1951, **28**, 434.
26. ZIMMERMAN, A. M., *Exp. Cell Research*, 1960, **20**, 529.