THE ISOLATION OF THE MITOTIC APPARATUS FROM MAMMALIAN CELLS IN CULTURE

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INTRODUCTION

Previous work has suggested that some protein(s) associated with the functioning of the mitotic apparatus (MA) in human amnion cells is synthesized during a relatively short period just prior to the entry of cells into division, and that this protein is conserved and reutilized by daughter cells during subsequent divisions (1). More direct evidence in support of this conclusion should be obtainable by biochemical analysis of isolated MA's. A method for isolating MA's from cultured mammalian cells is the subject of this report.

MATERIAL AND METHODS

Nearly all of the reports in the literature on the isolation of the MA concern the cleaving eggs of invertebrates. However, Pfeiffer (2) reported that the cold alcohol-peroxide-Duponol method of Mazia and Dan (3) was used to isolate the MA from grasshopper spermatocytes, and Stubblefield[1] has stated that the alcohol-digitonin technique of Mazia (4) can be made to work with mammalian cells in culture. This method has drawbacks (5), but the fact that it could be applied to mammalian cells indicated that perhaps one of the milder methods used for sea urchins might also be applied to mammalian cells. The method we present in this report is a modification of Kane's hexylene glycol technique (6). While this manuscript was in preparation, it was learned that Forer[2] has successfully used another modification of the Kane method for the isolation of the MA from cultured mammalian cells.

Most of our studies were carried out using the Fernandes line of human amnion cells (7), but HeLa S3 was also used with similar results. The general culture methods and media have been described (8, 9). Phase microscope observations indicated that the unmodified Kane isolation medium preserved spindle structure very well but did not lyse the cells. Lysis did occur, however, when phosphate was omitted from the medium and, while the mitotic apparatus could be obtained in the free and intact state, under these conditions it was stable for only 10–30 min at room temperature. Instability was characterized by shrinkage and loss of contrast in the phase microscope.

Since calcium ion has been shown to stabilize the MA (5, 10), calcium chloride was added at a concentration of 1×10^{-4} M to the isolation medium and at 2.5×10^{-4} M to a subsequent stabilizing solution. The lower concentration was chosen for the initial isolation because at concentrations above 1×10^{-4} M the MA's could not be freed from other cellular components. The higher $CaCl_2$ concentration was chosen for the second solution to further enhance stability. While calcium ion reduces the solubility of the isolated MA (5) and thus may limit the usefulness of the method for certain kinds of studies, we find that calcium-treated MA's are well suited for fine-structure studies as well as light and electron microscopic radioautography (unpublished findings).

The following is the detailed procedure. Mitotic cells collected according to the Robbins and Marcus (11) technique are sedimented at about 600 *g*, washed in Gey's balanced salt solution (sometimes omitted), and then resuspended in the isolation medium (1 M hexylene glycol, 1×10^{-4} M $CaCl_2$, pH 6.4–6.5). The cells are passed rapidly through a Pasteur pipette for 5–10 min to hasten lysis, and the released MA's are sedimented at about 130 *g*, and then resuspended in stabilizing solution (1 M hexylene glycol, 2.5×10^{-4} M, $CaCl_2$, pH 6.4–6.5). If it is observed at this stage that the degree of removal of cytoplasm is not sufficient, the MA's can be returned to the isolation solution for further treatment. All procedures are carried out at room temperature. As shown below, the MA can remain in the stabilizing solution for at least 20 hr at room temperature without visible degradation as seen in the phase microscope.

Phase photomicrographs were taken of MA's suspended in the stabilizing solution. For electron microscopy, MA's were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer containing 2.5×10^{-4} M $CaCl_2$ at pH 6.5 for 45 min, rinsed several times in

[1] E. Stubblefield. Personal communication.
[2] A. Forer. Personal communication.

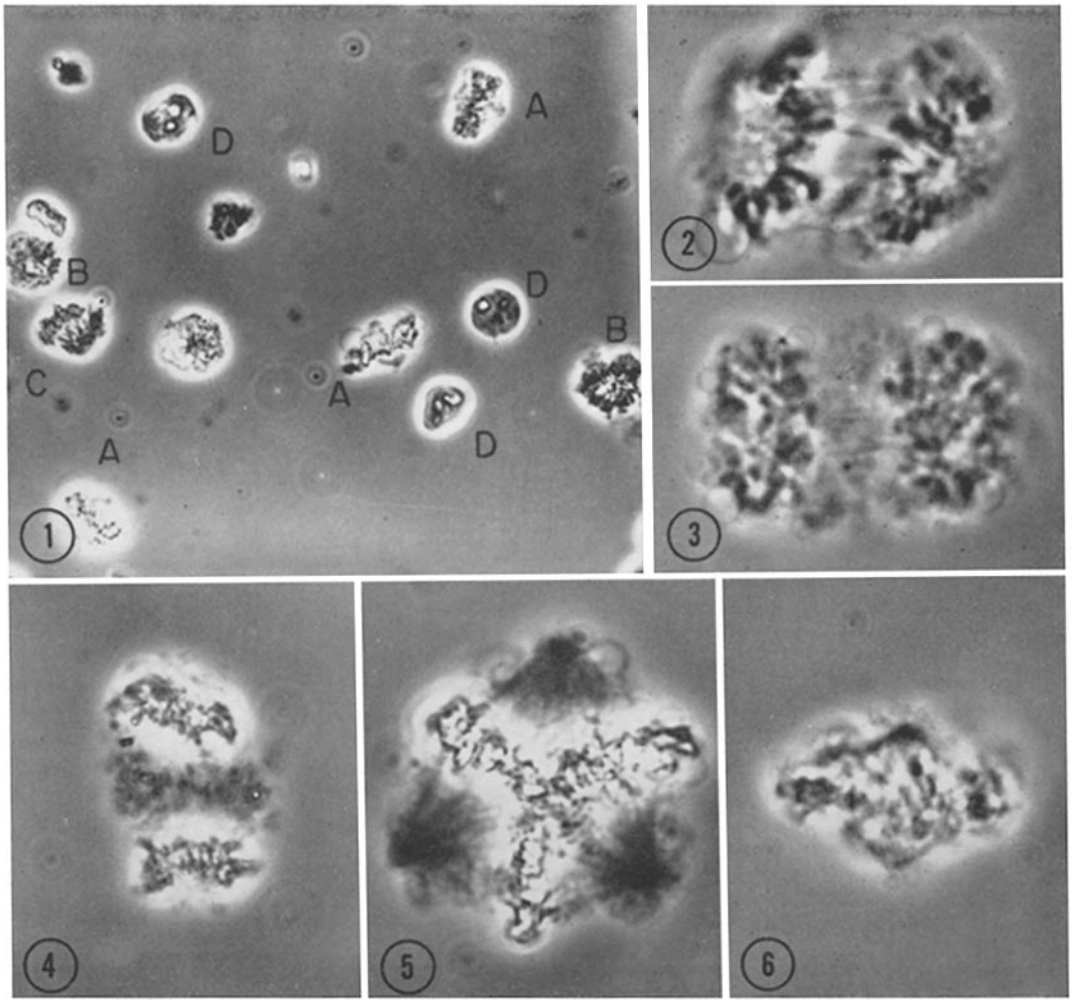

FIGURE 1 Low-power phase photomicrograph showing the general quality of preparations obtained. *A*, Metaphases oriented with spindles nearly parallel to plane of coverslip. *B*, Metaphases oriented with spindle nearly perpendicular to plane of coverslip. *C*, Anaphase. *D*, Interphase nuclei. × 410.

FIGURES 2–4 Anaphase figures showing retention of both fibrous and nonfibrous materials between the groups of separating chromosomes. Note the line of dense material across the equator of the mitotic apparatus in Fig. 4. Figs. 2 and 3, × 1750; Fig. 4, × 1200.

FIGURE 5 Tripolar metaphase. × 1700.

FIGURE 6 Normal metaphase. × 1750.

the same buffer solution, and postfixed in Caulfield's buffered osmium tetroxide with sucrose (12). The material was then dehydrated in a graded ethanol series, embedded in cross-linked methacrylate (13), and stained by Millonig's lead acetate-uranyl acetate staining technique (14).

RESULTS AND DISCUSSION

Fig. 1 illustrates, at low magnification, several features of the preparations we generally observe. It includes 3 metaphases (*A*) which are oriented with the spindles approximately parallel to the

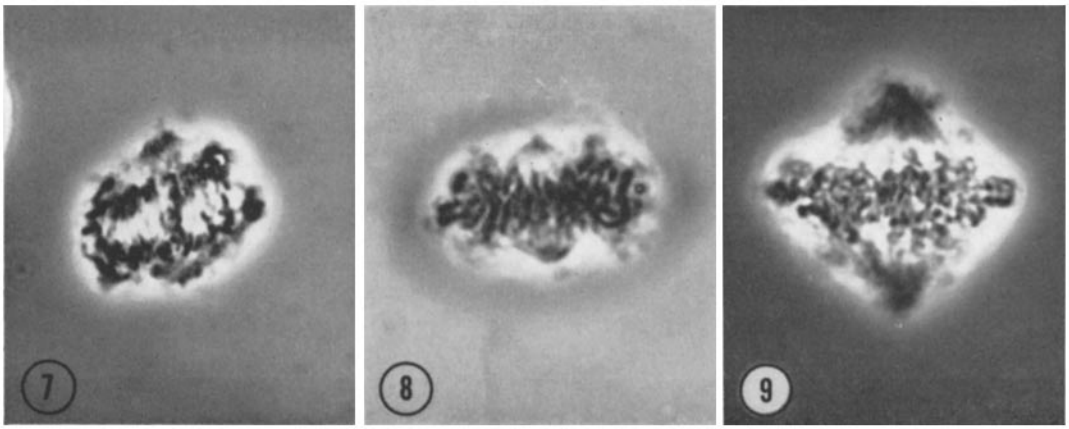

FIGURE 7 Anaphase which, after isolation, remained at room temperature for 2¼ hr before it was photographed. × 1200.

FIGURES 8–9 MA's which remained at room temperature for 20 hr before being photographed. Both MA's are from the same preparation. × 1600.

cover slip. However, as indicated by 2 examples (*B*), the metaphase MA may also be oriented with the axis of the spindle nearly perpendicular to the plane of the cover slip, rendering the spindle difficult to see. Floating the cover slip on an excess of the suspension often allows the MA to tumble, revealing that the two views are of the same objects. When the spindle is parallel to the cover slip, it is easily seen; when it rotates to a perpendicular position, it is nearly invisible. Fig. 1 also shows an anaphase (*C*) as well as some interphase nuclei (*D*). All of the structures are relatively free of cytoplasm. Since interphase nuclei are also freed of their cytoplasm by this treatment, the method might be useful for the gentle isolation of interphase nuclei in aqueous media.

Figs. 2–4 are anaphases which show fibrous material between the groups of chromosomes as well as a more amorphous ground substance. In Figs. 2 and 3, "thickening" of the spindle fiber in the equatorial region may be seen. In Fig. 4, the accumulation of material in this zone is much more obvious and conforms to what can be seen in fixed cells (e.g., 15). Fig. 5 shows an isolated tripolar metaphase of the type occasionally seen in cultured cells, and Fig. 6 is a normal metaphase. While, as shown in this photo, we sometimes see remnants of material attached to the spindle, we do not observe the kind of astral configuration seen in cleaving eggs, either in the fixed cell (16) or in the isolated MA. The photo of the anaphase in Fig. 7 was taken about 2¼ hr after isolation. Note that the upper pole, especially, looks like a double structure.

Figs. 8 and 9 are metaphases photographed after having been isolated and left in the stabilizing solution for 20 hr at room temperature. Although both MA's are from the same slide, there is a difference in the size and shape of the two spindles. The type of spindle in Fig. 8 is much more common; it tends to be ellipsoidal, and the pole-to-pole distance is shorter. This latter type might be a result of partial degradation.

As shown in a representative electron micrograph (Fig. 10), the microtubular elements of the spindle are not degraded by our isolation procedure. Kane and Forer (17) showed that isolated sea urchin spindles lost solubility during storage and that this loss of solubility was paralleled by degradation of spindle microtubules. However, it was not known whether these two phenomena were directly related. Since spindles isolated in the presence of calcium tend to be rather insoluble (5),[3] the preservation of microtubules in our material indicates that loss of solubility is not necessarily related to degradation of microtubules.

[3] G. M. Donnelly and J. E. Sisken. Data unpublished.

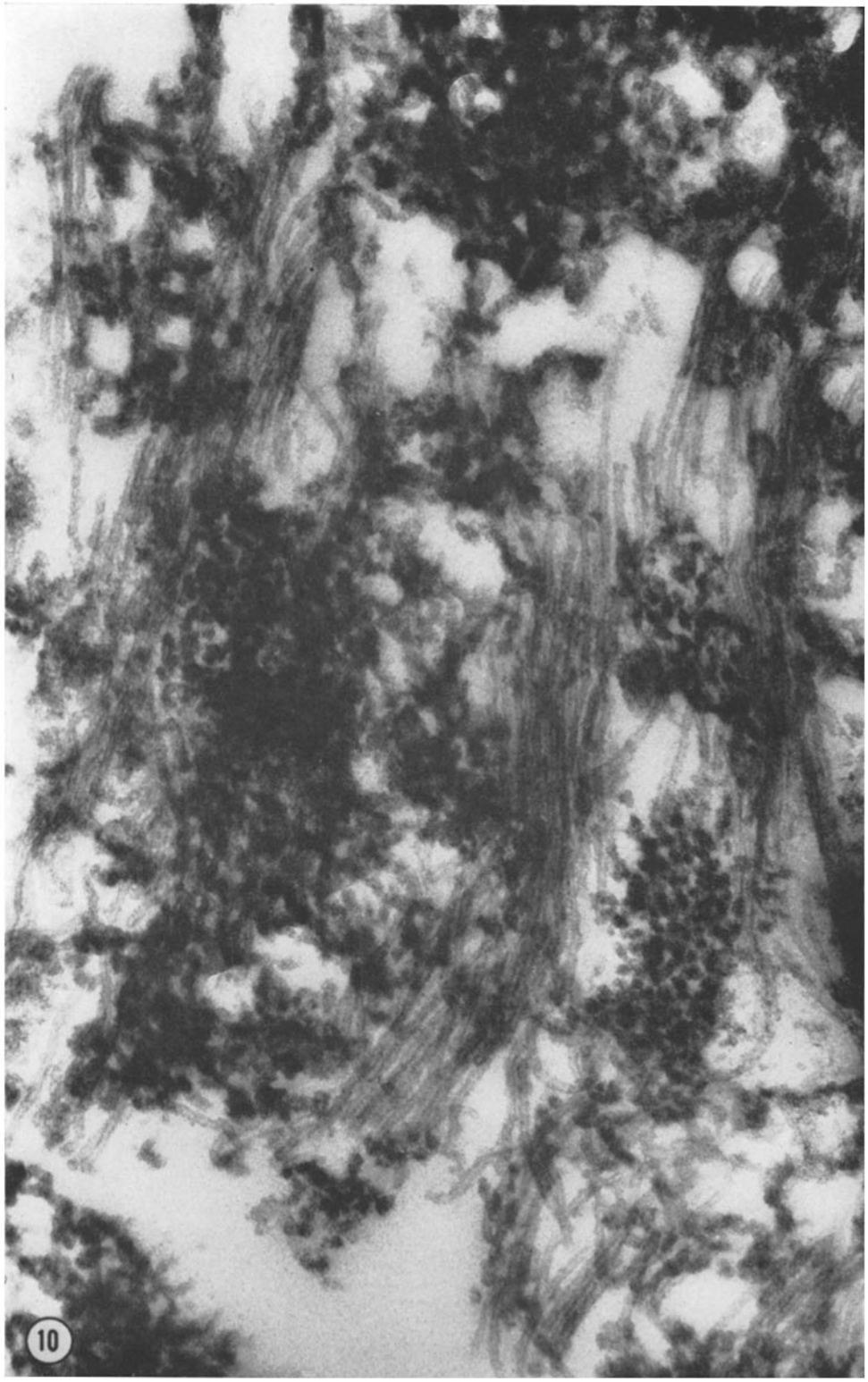

FIGURE 10 Electron micrograph of a portion of an isolated mitotic apparatus, showing intact spindle microtubules as well as the denser chromosomal substance. This material was exposed to the isolation medium for 30 min and to the stabilizing solution for 20 min prior to fixation. × 60,000.

SUMMARY

A method based on the Kane hexylene glycol procedure (6) has been developed for the isolation of the mitotic apparatus of mammalian cells in culture. The mitotic apparatus so isolated from human amnion and HeLa cells in culture retains cytological details commonly observed in cells fixed in toto.

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REFERENCES

1. SISKEN, J. E., and E. WILKES. 1965. *J. Cell Biol.* **27:** 97A. (Abstr.)
2. PFEIFFER, H. H. 1954. *La Cellule.* **56:** 243.
3. MAZIA, D., and K. DAN. 1952. *Proc Nat. Acad. Sci. U.S.A.* **38:** 826.
4. MAZIA, D. 1955. *Symp. Soc. Exptl. Biol.* **9:** 335.
5. MAZIA, D., J. M. MITCHISON, H. MEDINA, and P. HARRIS. 1961. *J. Biophys. Biochem. Cytol.* **10:** 467.
6. KANE, R. E. 1965. *J. Cell Biol.* **25:** 137.
7. FERNANDES, M. V. 1958. *Texas Rep. Biol. Med.* **16:** 48.
8. SISKEN, J. E., and R. KINOSITA. 1961. *J. Biophys. Biochem. Cytol.* **9:** 509.
9. SISKEN, J. E., and R. KINOSITA. 1961. *Exptl. Cell Res.* **22:** 521.
10. ROTH, L. E., and E. W. DANIELS. 1962. *J. Cell Biol.* **12:** 57.
11. ROBBINS, E., and P. I. MARCUS. 1964. *Science.* **144:** 1152.
12. CAULFIELD, J. B. 1957. *J Biophys. Biochem. Cytol.* **5:** 827.
13. KUSHIDA, H. 1961. *J. Electronmicroscopy.* **10:** 184.
14. MILLONIG, G. 1961. *J. Biophys. Biochem. Cytol.* **11:** 736.
15. KRISHNAN, A., and R. C. BUCK. 1965. *J. Cell Biol.* **24:** 433.
16. KLEINFELD, R. G., and J. E. SISKEN. 1966. *J. Cell Biol.* **31:** 369.
17. KANE, R. E., and A. FORER. 1965. *J. Cell. Biol.* **25:** 31.