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THE MITOTIC SPINDLE OF CHINESE HAMSTER OVARY CELLS ISOLATED IN TAXOL-CONTAINING MEDIUM

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SUMMARY

Mitotic spindles from Chinese hamster ovary (CHO) cells were isolated and purified by a one-step procedure in an isolation medium containing the microtubule-stabilizing drug, taxol. Released mitotic spindles were examined by phase-contrast, polarizing and differential-interference microscopy. They were also stained with monoclonal antibody raised against yeast tubulin and examined by epifluorescence microscopy. The spindles were free from visible cytoplasmic contaminants and the chromosomes were generally lost from the preparations. Electron microscopy showed that microtubules were the dominant structural component and sodium dodecyl sulphate/gel electrophoresis showed that tubulin was the major molecular species present, although a number of minor components, possibly representing microtubule-associated proteins (MAPs), were present. The taxol procedure was also useful in obtaining other microtubule-containing structures such as the midbody or the cytoplasmic microtubule complex in interphase cells. The taxol procedure was also used to isolate mitotic spindles from HeLa cells. The HeLa spindles stained positively with an antibody specific for the $210\times10^{3} M_r$ microtubule-associated protein, indicating that the MAP was retained by the taxol procedure. The taxol procedure appears to be of great advantage in large-scale preparations of spindles for biochemical analysis.

INTRODUCTION

Since the first successful isolation of the mitotic apparatus, achieved by Mazia & Dan (1952) over 30 years ago, several improved procedures have been developed to isolate more native mitotic spindles from sea-urchin eggs (Kane, 1965; Sakai & Kuriyama, 1974; Forer & Zimmerman, 1974; Rebhun, Rosenbaum, Lefebvre & Smith, 1974). Some of these methods have been adapted to other cleaving eggs or embryos of invertebrates (Rebhun & Sharpless, 1964; Bryan & Sato, 1970; Müller, 1970; Milsted & Cohen, 1973). In 1967, Sisken, Wilkes, Donnelly & Kakefuda first isolated mitotic spindles from cultured mammalian cells with the medium containing hexylene glycol originally devised by Kane (1965) for isolating sea-urchin spindles. Though this work was confirmed later (Wray & Stubblefield, 1970), bulk isolation and purification of mitotic spindles from mammalian cells with hexylene glycol as the microtubule-stabilizing agent proved to be much more difficult than from sea-urchin eggs. It was not until 1977 that the hexylene glycol technique was demonstrated to be useful for biochemical studies on the spindles of cultured mammalian cells (Chu & Sisken, 1977).

The hexylene glycol technique and, indeed, all the other spindle isolation methods

developed to date, have employed non-specific agents to stabilize the spindle. In addition to hexylene glycol, these include glycerol (Sakai & Kuriyama, 1974), dimethyl sulphoxide (Forer & Zimmerman, 1974), and polyethylene glycol (Kuriyama, 1982). These agents have in common the property of stabilizing microtubules, but, *a priori*, they would be expected to induce or stabilize many other protein–protein interactions as well. Since the microtubules of the mitotic spindle are immersed in the cytoplasmic milieu, these agents would be expected to generate adventitious associations between cytoplasmic components and the mitotic microtubules leading to contaminants being present in the isolated spindles. Clearly, a stabilizing agent that would be more specific for microtubules would be highly desirable.

Taxol, a macrocyclic alkaloid, appears to be such an agent. The drug stabilizes the structure of microtubules and enhances the *in vitro* polymerization of microtubules by decreasing the critical concentration for assembly (Schiff, Fant & Horwitz, 1979). It seems, therefore, to be an obvious choice as the stabilizer of mitotic microtubules during spindle isolation. One attempt to use taxol for spindle isolation has recently been reported (Zieve & Solomon, 1982), in which cultured mammalian mitotic cells were subjected to several centrifugations as well as being treated with DNase to release free mitotic spindles. In this paper, we describe our one-step procedure for the isolation of mitotic spindles from Chinese hamster ovary (CHO) cells with an isolation medium containing taxol, and present some general considerations for isolating mitotic spindles from tissue-culture cells.

MATERIALS AND METHODS

Cell culture and synchronization

Chinese hamster ovary cells were grown as monolayers in Ham's F-10 medium (Gibco Laboratories, Grand Island, N.Y.) supplemented with 5% foetal calf serum as described in a previous paper (Kuriyama & Borisy, 1981).

In order to obtain CHO cells blocked in mitosis, cells were first treated with 5 mM-thymidine. After 12–16 h, the cells were resuspended in fresh medium for 5 h, exposed to 0·1 μg/ml colcemid for an additional 3–4 h and then collected by centrifugation in a tabletop centrifuge (Kuriyama & Borisy, 1981).

HeLa cells were grown as monolayers in F-10 medium with 7% foetal calf serum and mitotic cells blocked by nocodazole were obtained according to the method described by Zieve, Turnbull, Mullins & McIntosh (1980).

Isolation of the mitotic spindles

In most experiments, 10^7 mitotic cells were used for each spindle preparation. All steps prior to cell lysis were carried out at 37 °C. Cells were released from the colcemid block by twice pelleting and resuspending in fresh culture medium without the drug. Recovery was continued for 15 min. Taxol (2 mg/ml of stock solution dissolved in dimethyl sulphoxide) was then added to a final concentration of 5 μg/ml (5·9 μM) to stabilize the mitotic spindles *in vivo* and the cells were continued in culture for 5 min more. The sample in a conical tube was spun in a tabletop centrifuge and then carefully washed by dropping distilled water down the side wall of the centrifuge tube without disturbing the pellet. After removing the washing liquid, the cells were suspended in 1 ml of isolation medium that contained 2 mM-PIPES (piperazine-*N*,*N*′-bis (2-ethanesulphonic acid)), 0·25% Triton X-100 and 20 μg/ml (23·4 μM) taxol at pH 6·8 and then gently disrupted by vortex-mixing.

Mitotic spindles from HeLa cells were isolated by the same procedure as for CHO spindles, except that recovery from nocodazole was extended to 30–35 min.

Isolation of the spindles was also performed on mitotic CHO cells in which the mitotic microtubules were not treated with taxol *in vivo*. That is, after recovery of the cells from colcemid for 20 min, spindles were isolated by directly resuspending the cell pellet with taxol-containing isolation medium as above. No morphological difference has been detected between spindles isolated from cells preincubated in taxol and those isolated from non-preincubated cells. Therefore, a 5-min treatment with taxol was not long enough to reorganize microtubule structures in cells.

To purify the spindles, the lysate was centrifuged at 3000 ***g*** for 15 min at room temperature, and the pellet was recovered.

Fluorescence staining

For staining the mitotic spindles with antibodies, samples of the isolated spindle fraction from CHO or HeLa cells were absorbed to polylysine-coated glass coverslips (Sato, Kuriyama & Nishizawa, 1983), and fixed in cold methanol or glutaraldehyde/$NaBH_4$ (Weber, Rathke & Osborn, 1978). After washing with phosphate-buffered saline (PBS: 8·0 g NaCl, 0·2 g KCl, 0·2 g KH_2PO_4, 0·99 g K_2HPO_4 in 1 litre), the coverslips were incubated with monoclonal anti-tubulin antibody raised against yeast tubulin (Kilmartin, Wright & Milstein, 1982) (generous gift from Dr J. V. Kilmartin, MRC Laboratory of Molecular Biology, Cambridge, England) or antisera against HeLa 210K microtubule-associated protein (MAP) according to the method described previously (Bulinski & Borisy, 1980). The coverslips were then incubated with fluorescein-labelled rabbit anti-rat immunoglobulin G (IgG) or goat anti-rabbit IgG (P. L. Cappel Laboratories, Inc.) for 1 h at 37 °C and washed in PBS for at least 3 h and mounted with Aqua-Mount (Lerner Laboratory, New Haven, Conn.) on microscope slides.

Microscopy

Light-microscopic observations were made with a Zeiss Universal microscope (Carl Zeiss, Oberkochen, FRG) equipped with epifluorescence optics, polarization and differential interference attachments.

Whole-mount electron microscopy of isolated spindles was performed as described previously (Kuriyama, 1982).

For preparation of thin sections, sedimented spindle fractions were fixed with 2·5 % glutaraldehyde in the microtubule polymerization buffer (0·1 M-PIPES, 0·5 mM-$MgSO_4$, 1 mM-EGTA (ethylene glycol-bis(β-aminoethylether)tetraacetic acid)). After postfixation with 1 % OsO_4, samples were stained with 0·5 % uranyl acetate, dehydrated through an ethanol series, infiltrated and embedded in an Epon formulation of Spurr according to the standard procedure described in a previous paper (Kuriyama & Borisy, 1981). Thin sections were stained with uranyl acetate and lead citrate and examined in a JEM 100S electron microscope operated at 80 kV.

Electrophoresis

Pellets of the mitotic spindles were resuspended and boiled in sodium dodecyl sulphate (SDS) sample buffer containing 10 % glycerol, 5 % 2-mercaptoethanol, 3 % SDS, 62·5 mM-Tris·HCl at pH 6·8, and analysed on SDS/polyacrylamide slab gels. Acrylamide gels (7½ %, w/v) were prepared according to the method of Laemmli (1970) and were run at a current of 35 mA and stained with Coomassie Blue by the procedure of Fairbanks, Steck & Wallach (1971). Molecular weight markers used were 94 000 (phosphorylase A), 68 000 (bovine serum albumin), 43 000 (ovalbumin) and 30 000 (carbonic anhydrase).

RESULTS

Observation of isolated spindles from CHO cells

The isolation of mitotic spindles essentially involves four separate processes: preparation by synchronization or differential selection of a pure mitotic cell

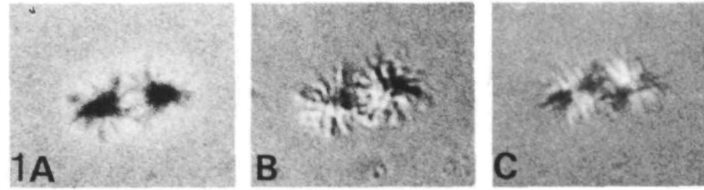

Fig. 1. Light micrographs of an isolated CHO spindle. The same spindle is shown in phase-contrast (A), differential-interference (B) and polarization (C) optics. ×1200.

population; stabilization of the spindle structure; cell lysis; and separation of the spindle from other cellular components. The choice of stabilizing agent is critical since it affects not only the stability of the spindle microtubules, but also the ease of cell lysis and the stickiness of the spindle for other cellular components.

Fig. 1A is a phase-contrast micrograph of a mitotic spindle isolated from CHO cells using a medium that contained taxol at 20 μg/ml. Since micromolar quantities of taxol are effective in stabilizing microtubules (Schiff *et al.* 1979), it is possible to disrupt mitotic cells completely in a low ionic strength medium, and the spindles were remarkably free from any visible cytoplasmic contaminants. The isolated mitotic spindle shows the typical shape of the spindle, and the poles appear to be connected to fibrous structures. With the low ionic strength medium used here, the chromosomes dissolved away following cell lysis, and did not appear in the isolated spindle fraction. Since the remaining kinetochore microtubules, as detected by electron microscopy of whole-mount preparations, had lost their attachment to chromosomes (data not shown), it is possible that the un-anchored microtubules sometimes splayed out to form an exaggerated configuration of spindle asters (Fig. 1).

Fig. 1B and C show the same spindle as in A, as revealed under polarizing and differential-interference microscopy, respectively. The differential-interference contrast image more clearly reveals the radial fibrous structure of the asters, as well as the fibrous structures connecting them. These features are further confirmed by the birefringence visible in the polarization image.

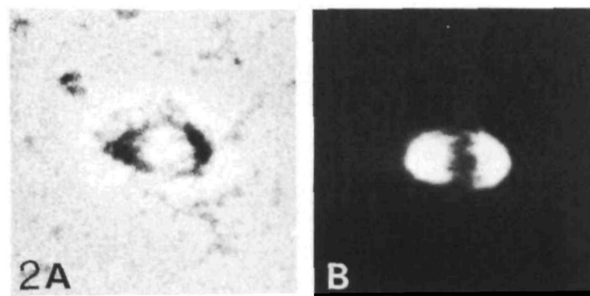

Fig. 2. Light micrographs of an isolated CHO spindle. The spindle was fixed with glutaraldehyde, reduced with sodium borohydride and indirectly stained with monoclonal anti-tubulin antibody. The micrographs are paired to show the same spindle after staining in phase-contrast (A) and fluorescence (B) microscopy. ×1300.

The isolated mitotic spindles from CHO cells were also visualized by immunofluorescence. Fig. 2 shows the phase-contrast (A) and the fluorescence micrograph (B) of the same isolated spindle together for comparison. In this case, the spindles were indirectly stained with monoclonal antibody against tubulin (Kilmartin *et al.* 1982), showing the localization of microtubules as expected from the fibrous framework of the spindle. It seems worthwhile to mention here that the phase-contrast image of the spindle differed slightly from that obtained with immunofluorescence microscopy. The mitotic microtubules, revealed by anti-tubulin antibody, extended significantly beyond the phase-dark areas located near the poles. This may suggest that the phase-dark appearance does not arise simply from the confluence of microtubules at the poles but may represent additional material as well, such as the centrioles and pericentriolar cloud.

Mitotic spindles isolated by previous techniques are fragile and mechanical shear tends to disintegrate them into half spindles (Kuriyama, 1982). The same seems to be true for the taxol-isolated spindles. Once the stabilization of mitotic microtubules was accomplished with taxol, the spindles were not temperature-labile and could be stored intact at 0°C. However, freezing and thawing of the isolates led to their disintegration.

Fig. 3 shows thin-section electron micrographs of the isolated fraction of mitotic spindles. The microtubules are one of the main components and it is easy to detect centrioles and pericentriolar material at the poles. However, it should be emphasized here that other structures, including some microfilaments and ribosome-like particles, were also detectable in our isolated fraction (Fig. 3B). Individual spindles isolated with taxol medium were also distinguishable by electron microscopy of whole-mount preparations, showing many microtubules attached to the virus particle-containing pericentriolar material (data not shown). Though the whole-mount preparation gave

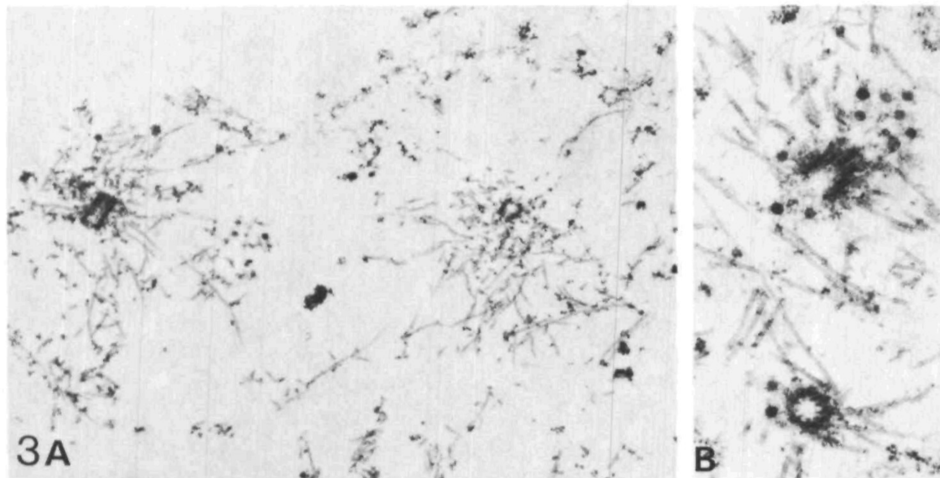

Fig. 3. Electron micrographs of the isolated CHO spindle fraction. The centrosomal area is shown in (B) at high magnification. Note the presence of some microfilament and ribosome-like structures in the isolates. A×13200; B×22200.

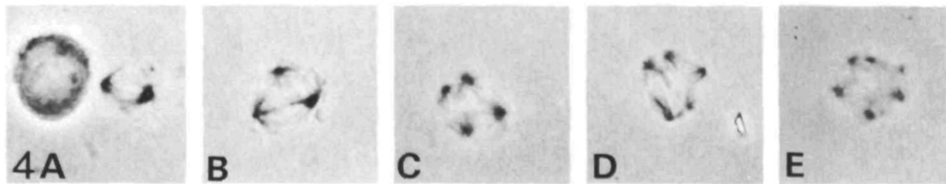

Fig. 4. Phase-contrast micrographs of isolated CHO multipolar spindles showing spindles with two (A), three (B), four (C), five (D) and six (E) poles. In A, the isolated nucleus is presented to compare its size with that of the spindle. ×820.

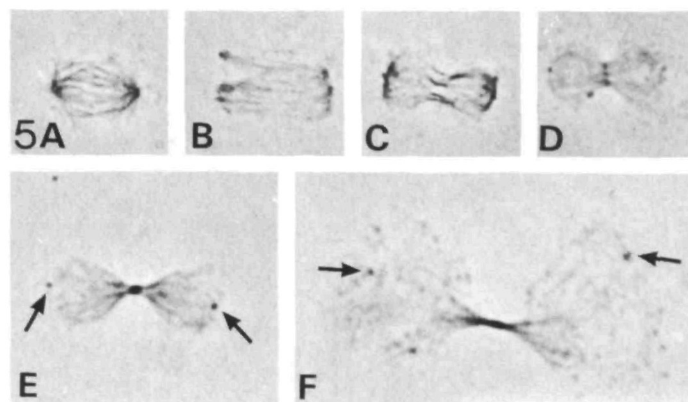

Fig. 5. Phase-contrast micrographs of the isolates showing various stages in the formation of the midbody and the cytoplasmic microtubule complex in daughter cells that are still connected to the midbody. Note the presence of small dots corresponding to the centrosomes (arrows in E and F). A, B, C, D×1180; E, F×970.

a better total view of the isolated spindle than the thin-sectioned material, the spindle image negatively stained with phosphotungstic acid, even with high-voltage electron microscopy, was of too high a contrast to print with moderate tone.

Observation of isolated mitotic spindles with phase-contrast microscopy revealed a variety of structures. Fig. 4 presents some of the spindle structures with multiple poles.

The isolation procedure described here for mitotic spindles is also applicable to microtubule-containing structures other than spindles. Fig. 5 illustrates stages in the formation of the midbody structure in a series of phase-contrast micrographs of the isolates. At the stage in which two daughter cells are shown to be still connected with a thin bridge of midbody (Fig. 5F), the cytoplasmic microtubule complex in interphase cells has started to become organized. It is possible to distinguish not only the microtubule system, but also dots (arrows in Fig. 5E, F) presumably corresponding to the centrosomes from which the cytoplasmic microtubules radiate. The structures seen in electron microscopy of the whole-mount preparations displayed the same shapes as seen in light microscopy and confirmed that the dots seen at the light-microscopic level corresponded to centrosomes. From other experiments with non-

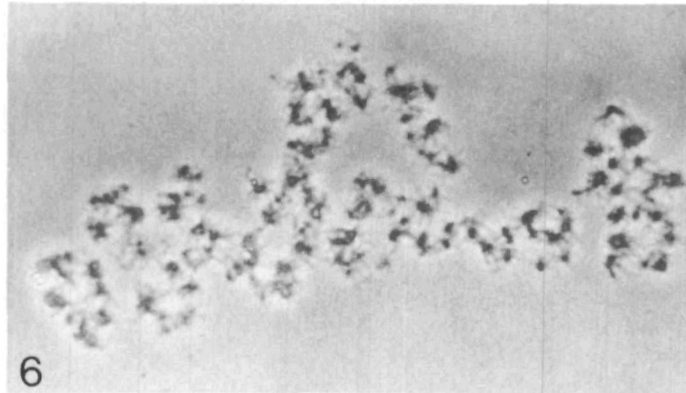

Fig. 6. Phase-contrast micrograph of mass-isolated spindles from CHO cells. ×910.

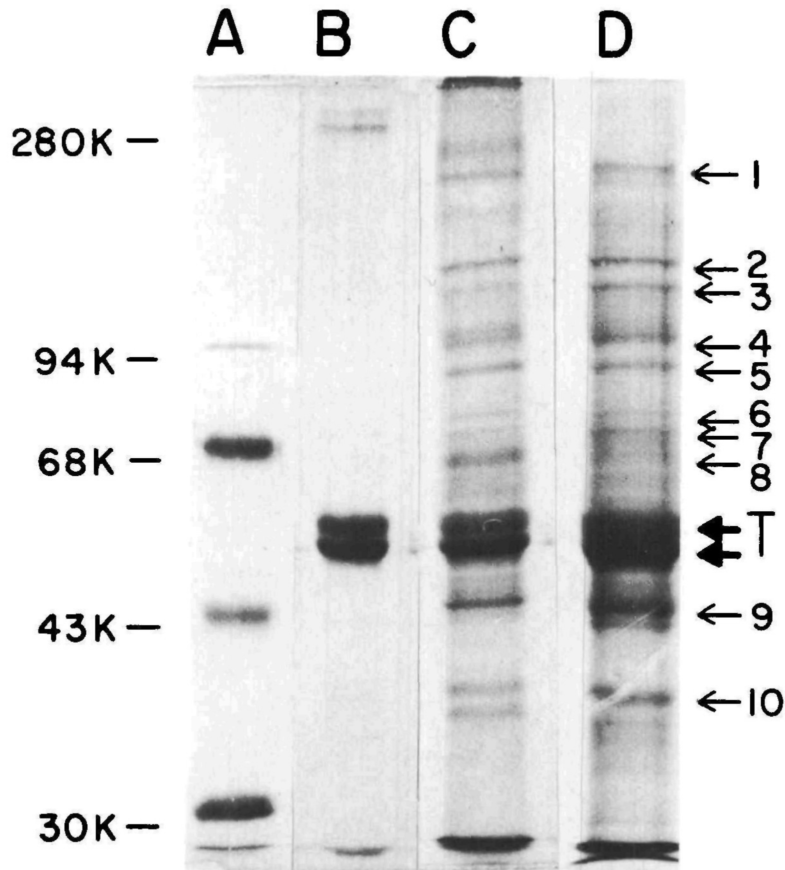

Fig. 7. SDS/polyacrylamide electropherogram of isolated CHO spindles. Lane A, molecular weight markers: 94 000, 68 000, 43 000 and 30 000. Lane B, porcine brain microtubule protein purified by two cycles of assembly and disassembly. Lanes C and D, two separate preparations of spindles. Small arrows indicate bands other than tubulin that consistently appear in spindle preparations; large arrows indicate tubulin (T).

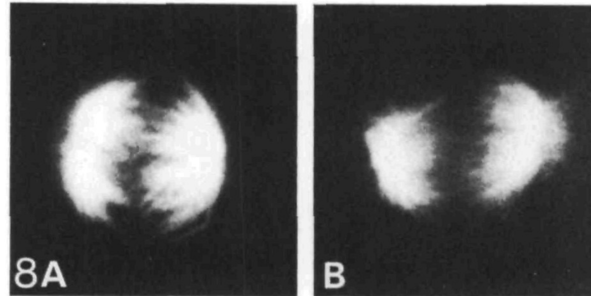

Fig. 8. Immunofluorescence micrographs of the isolated HeLa spindles stained with monoclonal anti-tubulin antibody (A) and anti-$210\times10^3 M_r$ MAP (B) antibodies. ×1300.

synchronized cell populations, it was also demonstrated that the cytoplasmic microtubule complex next to the nucleus could be visualized in taxol-extracted interphase cells.

Mass isolation of the mitotic spindles from CHO cells

The taxol-isolation procedure was readily adaptable for isolation and purification of mitotic spindles from CHO cells on a large scale. Fig. 6 shows a phase-contrast micrograph of mass-isolated mitotic spindles from CHO cells. Isolated spindles had a tendency to aggregate in suspension, so that micrographs of the resuspended fractions of the sedimented spindles gave an image of spindles sticking tightly together, rendering detailed visualization of individual spindles difficult. Specific staining with anti-tubulin confirmed that the aggregates were composed of numerous mitotic spindles (data not shown). The spindle fraction was clear and almost free from contaminants visible in the microscope. The only contaminants detectable at the light-microscopic level in this fraction were interphase nuclei and these were present at very low levels (~1 nucleus per at least 500 spindles).

We analysed the isolated CHO spindles by SDS/polyacrylamide gel electrophoresis, and sample electropherograms of the mitotic spindle proteins are shown in Fig. 7. As is evident, tubulin (T) is the dominant species, but a number of minor species are also present. Some of the characteristic minor species are indicated in Fig. 7 by arrows numbered 1–10. The pattern of minor species we detected appears similar to patterns obtained using the hexylene glycol procedure (Mullins & McIntosh, 1982).

We wondered whether the taxol-isolation procedure would retain specific microtubule-associated proteins along with the microtubules. The electropherograms of the isolated spindles did reveal numerous minor bands that could be candidates for MAPs; however, positive identification using a well-characterized antibody would be desirable. Such an antibody does not currently exist for CHO MAPs but one does exist for a $210\times10^3 M_r$ MAP present in cells of primates (Bulinski & Borisy, 1980). Therefore, we prepared spindles from HeLa cells using the taxol procedure and tested them for retention of the $210\times10^3 M_r$ MAP. Fig. 8 shows isolated HeLa spindles

immunostained either for tubulin (A) or $210\times10^3 M_r$ MAP (B) and the patterns indicate that the $210\times10^3 M_r$ MAP is retained and distributed throughout the isolated spindle as it is *in situ* (Bulinski & Borisy, 1980). Extrapolating from these results, it is reasonable to suggest that using the taxol procedure MAPs are also retained in spindles isolated from CHO cells.

DISCUSSION

Almost all of the studies on spindle isolation have been performed on dividing sea-urchin and other marine eggs, because they are particularly suitable for the large-scale collection of mitotic cell populations (Rebhun & Sarpless, 1964; Kane, 1965; Bryan & Sato, 1970; Sakai & Kuriyama, 1974; Forer & Zimmerman, 1974; Rebhun, Rosenbaum, Lefebvre & Smith, 1974; Salmon & Segall, 1980; Murphy, 1980); however, only a few studies have been carried out with other cell types, in particular, mammalian cells (Sisken *et al.* 1967; Wray & Stubblefield, 1970; Chu & Sisken, 1977; Kuriyama, 1982). It is of particular importance to establish a reproducible procedure for isolation and purification of mitotic spindles from cultured mammalian cells since a wealth of ultrastructural (Heath, 1981), functional (McIntosh, 1982), and genetic (Ling, 1981) information is now available and rapidly being expanded. It would be highly desirable to add to these approaches the capacity for biochemical analysis of the isolated spindles.

The major problem in the isolation of spindles from mammalian cells grown in culture arises from the antagonistic requirements for cell lysis and microtubule stabilization. The hypotonic solution necessary for cell lysis fails to preserve the microtubule structure. Further, the relatively non-specific reagents generally used for stabilizing spindle fibres, hexylene glycol or glycerol, make extraction of the cells difficult. On the other hand, the requirement of only micromolar quantities of taxol in stabilizing microtubules makes it possible to disrupt mitotic cells completely in hypotonic medium while preserving the integrity of spindle structure.

In the method described by Zieve & Solomon (1982) for isolating mammalian spindles, medium containing taxol and 0·1 M-PIPES was used as the lysis buffer and several steps of centrifugation and enzymic digestion were required to release free mitotic spindles into solution. The method described in this paper also uses taxol but differs in important details. A direct extraction of the mitotic cells in a low ionic strength medium facilitates the extraction of ground cytoplasm, chromosomes, membrane and surrounding intermediate filaments from the spindles in a quite simple and reproducible manner. This enables us to isolate the mitotic spindles in a pure fraction, providing significant advantage for large-scale preparations suitable for biochemical analysis.

In order to understand the detailed mechanism of mitosis, it is obviously necessary to know the molecular components of the spindle that might play an important role in controlling the mitotic function. Biochemical or immunolocalization experiments have already suggested the presence of ATPase and calmodulin (see Petzelt, 1979 for a review). Several kinds of MAPs were also demonstrated to exist as integral parts of

the spindle structure (Connolly, Kalnins, Cleveland & Kirschner, 1977, 1978; Sherline & Schiavone, 1978; Bulinski & Borisy, 1980). We were able to show that the $210\times10^3 M_r$ MAP in HeLa cells was retained in the spindles isolated with taxol-containing medium. There have been several efforts to identify the protein components specific to mitotic structures. Zieve & Solomon (1982) provided evidence from electrophoretic studies that a $150\times10^3 M_r$ protein associated with microtubules only during mitosis. More recently, Mullins & McIntosh (1982) defined a protein doublet of $115\times10^3 M_r$ molecular weight that was preferentially retained by the particulate fraction containing the matrix zone of the isolated midbodies. They also presented the overall protein components of the spindles isolated with hexylene glycol medium from CHO cells, showing over 35 bands of different intensities, which were similar to those shown in this paper (Fig. 7). As there is as yet no way to distinguish the specific proteins participating in the mitotic mechanism, identification of the minor and functioning components on gels remains to be resolved.

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