THE STRUCTURE AND SOME PROPERTIES OF
THE ISOLATED MITOTIC APPARATUS

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SUMMARY

The morphology of the isolated sea-urchin mitotic apparatus (MA) was examined by light and electron microscopy. With the polarization microscope and the Nomarski differential interference microscope, the isolated MAs appeared to be similar to in vivo MAs. Electron microscopy of the isolated MAs revealed the presence of microtubules, ribosome-like particles and vesicles. A close association between the ribosome-like particles and the MA microtubules resulted in the appearance of chains of particles running along the length of the microtubules.

Isolated MAs washed two to three times in isolation medium showed fine-structural changes in the electron microscope, which were reflected by lower retardation values obtained with the polarization microscope. The addition of magnesium and calcium or sucrose to the washing medium prevented these structural changes. Varying the pH of the isolation medium also resulted in changes in birefringence and ultrastructure of unwashed MAs.

Isolated MAs stored in the original isolation medium gradually became less birefringent and lost their microtubules. At pH 6.1 and pH 6.2 a residual birefringence was retained, even after several weeks of storage. Electron microscopy of these MAs revealed the presence of linear aggregates of ribosome-like particles oriented parallel to the long axis of the spindle. On the other hand, at pH 6.3 and pH 6.4, MAs lost their birefringence completely, and the ribosome-like particles became more randomly dispersed. 2M sucrose or 0.003M Mg²⁺ greatly retarded the loss of birefringence in stored MAs.

Glutaraldehyde-fixed MAs stained intensely with azure B bromide, demonstrating the presence of RNA. Treatment with RNase resulted in a loss of this staining. RNase-treated MAs examined with the electron microscope, revealed changes in the ribosome-like particles.

The results are discussed in the light of recent biochemical analyses of the isolated MA, structural similarities to in situ MAs and the interpretation of the birefringence of the MA.

INTRODUCTION

For many years the existence of fibres in the mitotic apparatus (MA) was a source of controversy (Wilson, 1928; Heilbrunn, 1943; Schrader, 1953; Mazia, 1961). In living material viewed with the bright-field or phase-contrast microscope, the MA appears as a clear region with little internal fibrous structure (Wilson, 1928; Heilbrunn, 1943; Schrader, 1953), although fibres can be seen in the asters of living marine eggs, due to the association of cytoplasmic particles along their length (Heilbrunn, 1943). In fixed material, or in living cells treated with acid medium (see Lewis in Schrader, 1953), fibres are easily recognizable in the MA, but the possibility exists that fixation or acid-treatment of living cells causes coagulation of proteins within the spindle to give the appearance of fibres.

Schmidt (1937, 1939) reported that spindles in living cells showed positive bire-
fringence with respect to their long axes and, although no fibres were observed, he concluded that oriented protein units were responsible for the birefringence of the MA. Several other workers have confirmed these observations (Hughes & Swann, 1948; Swann, 1951a, b; Inoué & Dan, 1951). In 1953 Inoué presented the first definitive evidence for the existence of spindle fibres in living cells using an improved polarization microscope (Inoué, 1951, 1952). More recently, Bajer & Allen (1966) have observed spindle fibres in living Haemanthus cells with the Nomarski differential interference microscope. It appears, therefore, that a major component of the mitotic apparatus in living cells is a set of birefringent fibres, positive in sign with respect to the long axis of the spindle.

Electron-microscopic observations of cells undergoing mitosis have revealed the presence of individual microtubules and bundles of microtubules in the MA. Ranging in diameter from 180 Å to 280 Å, the microtubules have been reported in plant cells (Leak & Wilson, 1962; Pickett-Heaps & Northcote, 1966; Roth, Wilson & Chakraborty, 1966), fibroblasts (Krishan & Buck, 1965), crayfish spermatocytes (Ruthmann, 1959), amoeba (Roth, 1964; Roth & Daniels, 1962), ciliates (Jenkins, 1967), insect spermatocytes (Roth et al. 1966), and sea-urchin eggs (Gross, Philpott & Nass, 1958; Harris, 1962, 1965). It is reasonable to suggest that the microtubules seen with the electron microscope correspond to the birefringent fibres seen with the light microscope. This hypothesis has been supported by evidence presented by Rebhun & Sander (1967), although denied by that reported by Behnke & Forer (1966).

In 1952, Mazia & Dan showed that MAs could be isolated from sea-urchin eggs by a technique involving fixation in cold ethanol. Later, Mazia and his co-workers (Mazia, Mitchison, Medina & Harris, 1961) showed that MAs could be isolated directly from living eggs (i.e. without prior fixation) using the disulphide reagent, dithiodiglycol (DTDG). Kane (1962a) has since shown that an –S–S– reagent is not necessary for the isolation of the MA. He demonstrated that penetrating non-electrolytes, such as propylene glycol, can be used as successfully as DTDG in the isolation medium.

Morphologically, isolated MAs appear fibrous in the phase-contrast microscope (Mazia & Dan, 1952; Kane, 1962a), birefringent in the polarizing microscope (Mazia & Dan, 1952; Kane & Forer, 1965; Rebhun & Sander, 1967) and possess microtubules when viewed with the electron microscope (Kane, 1962b; Rebhun & Sander, 1967; Sisken, Wilkes, Donnelly & Kakefuda, 1967). Thus, in general, they show a close morphological similarity to the in situ MA.

Since changes of birefringent material in in vivo MAs have been used to infer possible mechanisms by which it may function (e.g. Schmidt, 1939; Mitchison & Swann, 1952; Inoué, 1964; Forer, 1966), it is most important to understand thoroughly the structures which give rise to birefringence in the MA and the conditions which may cause modification of this birefringence. For this reason we have undertaken a study of the birefringence of the isolated MA under a variety of experimental conditions and have correlated it with electron-microscope observations. The results indicate that slight changes in the environment of the isolated MA produce recognizable changes in its ultrastructure and in its birefringent properties.
**MATERIALS AND METHODS**

*Arbacia punctulata* were supplied on a year-round basis by Mr Glendle Noble of Panama City, Florida, U.S.A. Eggs were shed from ripe females by the application of voltage across the sea-urchin test (Harvey, 1956), and sperm preparations were made by diluting ‘dry’ sperm with sea water.

**MA isolation procedure**

Eggs were washed 4–5 times in artificial sea water; 1–3 ml of eggs were suspended in 100 ml of sea water maintained at 20 °C and fertilized with a 1 % sperm suspension. Thirty seconds after fertilization 100 ml of calcium-free sea water (20 °C, pH 7.8–8.0) containing 1–2 mg/ml of mercaptoethyl-gluconamide was added (Mazia *et al.* 1961). Ten minutes later the fertilization membranes were removed by passing the eggs through finely meshed bolting cloth, and the eggs were then concentrated by spinning in a hand-centrifuge. The supernatant was removed, and the eggs were resuspended in 100 ml of fresh calcium-free sea water. This operation was repeated, and the eggs were allowed to develop in Ca2+-free sea water maintained at 20 °C. When the eggs began to form the first cleavage spindle, they were sedimented in a hand-centrifuge and resuspended in a 0.5 M NaCl:0.5 M KCl (19:1) mixture (Kane, 1962a). The eggs were again concentrated and resuspended in 10–15 vol. of isolation medium. The eggs were allowed to stand for 2 min at 20 °C, after which the suspension was shaken in a chilled Erlenmeyer flask. This final step aided in the mechanical dispersion of the egg cytoplasm and the subsequent release of the MAs. Once the MAs were isolated and chilled, all subsequent operations were carried out at 0–4 °C.

**Isolation medium**

The isolation medium was identical to Kane’s (1965) medium. It consisted of 12 % hexylene glycol (about 1.0 M) in 0.1–0.01 M KH2PO4-NaOH buffer at pH 6.1–6.4. MAs were washed by alternate low-speed centrifugation and resuspension in fresh aliquots of this isolation medium. In several experiments, 2 M sucrose or 0.003 M MgCl2 was dissolved in the medium.

**Light-microscope observations**

MAs were observed either in isolation medium or, if fixed, in distilled water. Most polarized-light observations were made with the American Optical Ultra-Bio Polestar microscope, fitted with rectified condenser and objectives. The instrument was equipped with a 22-mμ compensator. An Osram HBO 200 mercury arc light-source was utilized for all polarized light microscopy. The light was passed through a heat filter for cooling and then a mercury green interference filter.

Phase-contrast observations were made with either a Zeiss Winkel or Reichert phase-contrast microscope, with a tungsten-filament light source. The Zeiss Autophot microscope, with a zirconium-arc light source was used for Nomarski differential interference observations.
Measurements in polarized light

For all measurements in polarized light, the isolated MAs were placed with their long axis at 45° to the crossed polars. MA retardations were determined by the maximum darkening method of Bear & Schmitt (see Bennett, 1950). The formula used for converting compensator angle settings to retardation (\( \Gamma \)) was:

\[
\Gamma_0 = - \Gamma_c \sin 2C_1,
\]

where \( \Gamma_0 \) = object retardation, \( \Gamma_c \) = maximum compensator retardation, \( C_1 \) = compensator setting at point of maximum darkening of object. The retardation values recorded for each MA were the average of two readings in adjacent quadrants of the microscope field. Measurements were made by observing maximum darkening of the brightest part of the chromosome-to-pole region.

Electron microscopy

MAs were fixed in either 1% OsO\(_4\) or 2.5% glutaraldehyde followed by 1% OsO\(_4\) in isolation medium. pH adjustment was made after the glutaraldehyde was added to the isolation medium. MAs were spun out of the medium at low speed and after decanting most of the supernatant, were resuspended in the fixation medium.

For determining the immediate effect of sucrose on MA ultrastructure, MAs were spun out of the egg lysate and resuspended in 2 m sucrose in isolation medium for 1 h in the cold. Following this, the MAs were again centrifuged and resuspended in 2.5% glutaraldehyde in isolation medium containing 2 m sucrose. The identical procedure was followed for MAs stored in sucrose for longer periods.

For electron-microscope observations of magnesium effects, the MAs were fixed in 1% OsO\(_4\) in isolating medium containing 0.003 M MgCl\(_2\).

All MAs were fixed at 0-4 °C for at least 1-2 h, and in the case of OsO\(_4\) were sometimes allowed to remain overnight in fixation medium. Following fixation, MAs were rinsed twice in fresh isolation medium and twice in distilled water. The MAs were then dehydrated in an alcohol series, and embedded in Epon 812 (Luft, 1961).

The blocks were sectioned with a Cambridge Huxley microtome or a Sorvall MT-2 microtome, using a diamond knife. Sections were mounted on Formvar/carbon-coated copper grids and were stained with uranyl acetate (Stempack & Ward, 1964) followed by lead citrate (Reynolds, 1963). Observations were carried out with an Hitachi HS-7s microscope utilizing a 200 µ condenser aperture and 20 or 50 µ objective apertures.

Cytochemistry

MAs fixed in glutaraldehyde for 1 h or less were employed for both light and electron cytochemistry. For light microscopy, RNA was detected by the azure B bromide technique described by Swift (1955). RNase digests were carried out for 1-2 h in distilled water containing 1 mg/ml RNase (Sigma, 5x cryst.) at pH 6.0-6.5, and maintained at room temperature (Swift, 1955). All controls for the enzyme digestions were carried out under identical conditions, excluding the enzyme.
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Following enzyme digestion, MAs used for electron microscopy were post-fixed in 1 \% OsO$_4$ in distilled water for 1 h. These MAs were then dehydrated and embedded.

Photography

Light micrographs were taken on Tri-X (Kodak), Plus X (Kodak), or KB-14 (Adox) film. Light measurements were made with a photo-volt photometer. Films were developed with Diafine two-stage developer (Baumann Photochemical Corp., Chicago, Ill.).

Statistical analyses

Retardation data from isolation experiments at different pH values were subjected to an analysis of variance (Table 1) with the aid of Dr S. Taub, Department of Biology, Princeton University. From the data obtained, a comparison among the means was carried out according to Snedecor (1956).

For Tables 2 and 3, standard deviations (s.d.) were calculated according to the formula

$$S.D. = \left( \frac{\Sigma (x^2) - \left(\frac{\Sigma x}{n}\right)^2}{n-1} \right)^{1/2}$$

where $x$ = individual MA measurements, and $n$ = total number of MA measurements.

Tables 4 to 11 utilize the two-tailed $t$ test, where

$$t = \left( \frac{X_i - X_j}{\frac{n_1 n_2 (n_1 + n_2 - 2)}{(n_1 + n_2) \left(\frac{\Sigma x^2}{n} + \frac{\Sigma y^2}{n_2}\right) - \frac{\Sigma x^2}{n} - \frac{\Sigma y^2}{n_2}} \right)^{1/2}$$

$X_i$, $X_j$ = mean values for two series of MA $\Gamma$ values, $n_1$, $n_2$ = total number of MA retardation values for each series, and $X_i$, $X_j$ = individual MA $\Gamma$ values.

The value for $P$ (probability) found in Tables 4–11 was determined from standard statistical tables.

RESULTS

General morphology of the isolated mitotic apparatus

All of the following morphological descriptions refer to the freshly liberated MA observed before washing away the cytoplasmic debris. To reduce variability caused by differences in retardation at various stages of mitosis, all polarized light measurements were made on metaphase MAs.

The spindle fibres of the MA are seen with phase, Nomarski and polarization optics (Figs. 1–4). The most conspicuous of these are the chromosomal fibres, which run from the chromosomes to the poles. In polarized light these are seen as thicker regions of higher retardation (Fig. 3). Continuous, or pole-to-pole fibres are not easily seen, but they presumably contribute to the birefringence of the non-chromosomal fibre portion of the spindle body (Figs. 3, 4). The astral fibres are best revealed in polarized light, where they form a ‘maltese cross’ with the centrosomal region at the centre (Fig. 3). A fringe of isotropic cytoplasm completely surrounds the birefringent elements of the MA (Fig. 3).
At low magnification in the electron microscope (Fig. 5), the spindle appears to consist of fibres, ribosome-like particles, randomly distributed vesicles, and very dense granular chromosomes. Segments of continuous fibres are seen to run between the chromosomes.

Higher-power micrographs demonstrate the microtubular nature of the fibres. Single microtubules and bundles of two or more closely packed microtubules are observed in both longitudinal and cross-sections (Figs. 6–9). The microtubule diameters range from 240 Å to 280 Å, and the dense outer walls range in thickness from 40 Å to 90 Å. A suggestion of substructure in the walls of microtubules is seen in high-resolution micrographs of cross-sections of MAs, although this is difficult to determine with certainty.

The microtubules are generally directed along the long axis of the spindle (Figs. 6, 7). They may be perfectly straight (Figs. 6–8), gently curved (Figs. 6–8) or kinked (Figs. 7, 8).

The ribosome-like particles range in diameter from 230 Å to 270 Å and the majority appear to be distributed around the microtubules (Figs. 5–8) in a rather haphazard manner, leaving some regions of the microtubule surface completely 'naked', and other regions densely packed with particles (Fig. 7). In some instances, chains of particles are seen connecting separate microtubules (Fig. 7). The particles also appear to be associated with an amorphous material (Fig. 8).

Chromosome-microtubule associations are found in all e.m. preparations, although no definitive morphological entity, such as a kinetochore, is found at junctures of microtubules and chromosomes. Some microtubules stop at the chromosome surface, while others seem to pass through the chromosome or immediately adjacent to it (Fig. 10).

A typical centriole (Fig. 11), measuring approximately 4500 Å in diameter, is found at the centre of the astral region. The remainder of the astral region consists of longitudinal and cross-sections of microtubules, which is to be expected since birefringent fibres radiate from the centrosomal region.

Most of the vesicles of the MA are spherical in shape (Figs. 5–10), and appear in a variety of sizes. Some contain small amounts of fibrous material, and others contain a material of low density filling the entire lumen (Fig. 10). All the vesicles are membrane-bounded, and most are smooth-surfaced.

The vesicles are not seen in MAs of fixed whole cells. It seems likely that the vesicles seen in isolated MAs are elements of the smooth endoplasmic reticulum (Kane, 1962b; Rebhun & Sander, 1967), which become swollen in the hypotonic isolation medium. However, a few of the vesicles of the isolated MA appear to have ribosome-like particles associated with them (Fig. 7). Rebhun & Sander (1967) have also seen some rough-surfaced endoplasmic reticulum in electron micrographs of isolated Spisula MAs.

Effects of the pH of the isolation medium on the isolated MA

The pH range chosen for investigation was pH 6·1–6·4, since the various MA isolation methods for marine eggs have all used pH values within this range (Mazia et
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Prior to isolation the eggs were allowed to develop at 20 °C in a carefully controlled temperature bath, as it was found that even a slight change (1–2 °C) in the temperature of developing eggs altered the retardation values of the isolated MAs. All isolations in these experiments were also carried out at 20 °C. Retardation measurements were made on unwashed MAs in the isolation lysate, within 15 min of isolation.

The MAs at all pHs tested appeared similar in general morphology when observed with phase-contrast and polarized light (Figs. 1, 3, 4). However, the retardation measurements revealed some important pH-dependent differences. The data for this series of experiments are found in Table 1. In each experiment, measurements were made on ten different metaphase MAs. An analysis of variance was performed on the pooled data in Table 1, and it was found from the F ratios that there is a significant effect of pH on retardation. In addition to this the data demonstrate that there is also a significant difference from one experiment to the next at each pH. This difference may arise from physiological variations between batches of eggs from different females.

A comparison among means (see Snedecor, 1956, p. 221) tells us where the differences among the pH values lie (Table 1). At the upper 1 % level of confidence it is found that retardation measurements on MAs isolated at pH 6·2 and 6·3 do not differ significantly from each other, but do differ from measurements on MAs isolated at pH 6·1 and 6·4, which also differ significantly from one another. By inspection of the data in Table 1 one can readily see that the pH 6·1, 6·2 and 6·3 values are quite similar, and that the pH 6·4 values differ dramatically from the others.

The possibility that these effects are due to swelling or shrinking of the birefringent region of the isolated MAs was checked by measuring the length and width of the birefringent spindle body at each of the four pH values. The data presented in Table 2 indicate that there is no difference in size, and thus variation in size cannot account for the observed variations in retardation.

Table 1. pH effects on retardation of unwashed MAs isolated at 20 °C

<table>
<thead>
<tr>
<th>pH of isolation medium</th>
<th>No. of experiments</th>
<th>No. of measurements</th>
<th>Average retardation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>6·1</td>
<td>6</td>
<td>60</td>
<td>48</td>
</tr>
<tr>
<td>6·2</td>
<td>4</td>
<td>40</td>
<td>53</td>
</tr>
<tr>
<td>6·3</td>
<td>3</td>
<td>30</td>
<td>51</td>
</tr>
<tr>
<td>6·4</td>
<td>8</td>
<td>80</td>
<td>32</td>
</tr>
</tbody>
</table>

Analysis of variance: F Ratios, pH/expts = 15·9, expts./measurements = 3·3; P for both ratios < 0·01.

From data calculate a comparison among means (see Snedecor (1956), p. 221). At the upper 1 % confidence level, pH 6·2 and 6·3 are not significantly different from each other, pH 6·1 and 6·4 are significantly different from each other, and pH 6·2 and 6·3 are both significantly different from pH 6·1 and from pH 6·4.

* Values are in Angstroms.
As yet no counts have been made of numbers of microtubules in MAs isolated at different pHs. However, no indications of differences in microtubule numbers have been seen. The only ultrastructural difference between MAs isolated at pH 6.1 and those isolated at pH 6.4 seems to lie in the association between microtubules and ribosome-like particles. Most of the ribosome-like particles are closer to the walls of the microtubules at pH 6.1 (see, for example, Fig. 6), while a good proportion of the particles are further away from the microtubule walls at pH 6.4 (Fig. 12). The latter morphology gives the appearance of a more random distribution of particles around the microtubules. The higher retardations obtained at pH 6.1 may be due, in part, to the higher degree of orientation of ribosome-like particles caused by their closer association with the microtubules. Conversely, the more random distribution of particles at pH 6.4 might contribute to the lower retardation readings. This would agree with the evidence that the majority of MA birefringence is form birefringence (Rebhun & Sander, 1967) and thus changes in the degree of orientation of the form birefringent components might cause changes in the measured retardation.

**Effect of pH on stability of birefringence and ultrastructure**

Isolated MAs stored in the original isolation lysate at 0-4 °C gradually lose their birefringence. This loss of birefringence is dependent upon the pH of the isolation medium.

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### Table 2. Average spindle size at each pH tested

<table>
<thead>
<tr>
<th>pH of isolation medium</th>
<th>No. of measurements</th>
<th>Average width* (μ)</th>
<th>Average length† (μ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1</td>
<td>10</td>
<td>10.4 (1.2)</td>
<td>19.9 (1.29)</td>
</tr>
<tr>
<td>6.2</td>
<td>10</td>
<td>11.2 (0.79)</td>
<td>18.8 (0.92)</td>
</tr>
<tr>
<td>6.3</td>
<td>10</td>
<td>10.3 (1.83)</td>
<td>18.9 (0.99)</td>
</tr>
<tr>
<td>6.4</td>
<td>10</td>
<td>10.7 (0.68)</td>
<td>19.5 (0.97)</td>
</tr>
</tbody>
</table>

* Width = spindle width (width of birefringent region at the metaphase plate).
† Length = spindle length (length of birefringent region from pole to pole).
Values in parentheses = standard deviation.

### Table 3. Decrease in retardation (Γ) of MAs stored at 0-4 °C

(A comparison of the mean retardation values of freshly isolated MAs with stored MAs.)

<table>
<thead>
<tr>
<th>pH of isolation medium</th>
<th>No. of measurements</th>
<th>Average retardation values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0-15 min</td>
</tr>
<tr>
<td>6.1</td>
<td>10</td>
<td>43 (6.3)</td>
</tr>
<tr>
<td>6.1</td>
<td>10</td>
<td>49 (4.4)</td>
</tr>
<tr>
<td>6.1</td>
<td>10</td>
<td>59 (11.6)</td>
</tr>
<tr>
<td>6.2</td>
<td>10</td>
<td>49 (6.0)</td>
</tr>
<tr>
<td>6.2</td>
<td>10</td>
<td>66 (6.9)</td>
</tr>
</tbody>
</table>

Values in parenthesis = standard deviation.
After storage for 24 h, pH 6.1 and 6.4 MAs still possess a detectable birefringence, but this is too weak to measure accurately. After 48 h the birefringence disappears completely. pH 6.1 and 6.2 MAs, on the other hand, maintain a measurable residual birefringence after 120 h (see Fig. 13 and Table 3).

MAs isolated and stored at pH 6.3–6.4 for 12–24 h were fixed and subsequently observed with the electron microscope. There were no recognizable microtubules in these MAs (Fig. 14). On rare occasions, very short, linear arrays of ribosome-like particles and fibrous material were seen, but there were no microtubules associated with them. Furthermore, the ribosome-like particles seemed to be much more randomly distributed than was observed in MAs fixed immediately after isolation.

At low magnification the ultrastructure of MAs isolated and stored at pH 6.1–6.2 for 48–120 h appeared similar to that of freshly isolated MAs. At higher magnification the 'spindle fibres' were seen to consist of linear arrays of ribosome-like particles, but intact microtubules were missing (Fig. 15). There was some other electron-dense material present, which may represent microtubule breakdown products (Fig. 16). In a few instances, what appeared to be remnants of microtubules were observed.

The observation that the birefringence of stored pH 6.4 MAs gradually disappears was first made by Kane & Forer (1965). However, these workers used washed pH 6.4 MAs, which were stored at room temperature. In the electron microscope, MAs treated in this manner show a linear accumulation of dense material not associated with ribosome-like particles. Kane & Forer (1965) concluded that this material is a microtubule breakdown product. Such material has not been seen in the pH 6.4 MAs observed in this study, but there was some indication of a similar material in stored MAs isolated at pH 6.1–6.2 (see md in Fig. 16).

Prolonged maintenance of structural integrity by the use of sucrose

It was found that MAs stored in isolation medium containing 2 M sucrose retained their birefringence for much longer periods of time. Table 4 contains the retardation data for MAs stored in sucrose and observed after various lengths of time. The experiments show that there is no significant reduction in retardation for up to 288 h following isolation.

<table>
<thead>
<tr>
<th>pH of isolation medium</th>
<th>No. of measurements</th>
<th>Average retardation values at 0–30 min</th>
<th>84 h</th>
<th>90 h</th>
<th>288 h</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1</td>
<td>10</td>
<td>21</td>
<td>22</td>
<td>—</td>
<td>—</td>
<td>&lt; 0.7</td>
</tr>
<tr>
<td>6.1</td>
<td>10</td>
<td>18</td>
<td>—</td>
<td>—</td>
<td>16</td>
<td>&lt; 0.7</td>
</tr>
<tr>
<td>6.4</td>
<td>10</td>
<td>15</td>
<td>13</td>
<td>—</td>
<td>—</td>
<td>&lt; 0.7</td>
</tr>
</tbody>
</table>

P* = probability (see Materials and Methods).

Retardation measurements made on MAs in the sucrose solution are much lower than those of corresponding unwashed MAs measured in the normal isolation medium. This is probably
due to the higher refractive index of the sucrose solution, and this would be consistent with the observation that the major portion of the isolated MA birefringence is form birefringence (Rebhun & Sander, 1967; Pfeiffer, 1952). To make certain that the sucrose effect on retardation is reversible and is probably due to a change in refractive index of the medium, MAs were treated with 2M sucrose for one hour and then resuspended in fresh isolation medium. A set of measurements was then made in polarized light. Table 5 presents data for pH 6.1 and pH 6.4 MAs, before and after sucrose treatment which show no significant change due to this treatment. The sucrose-treated MAs appeared identical to freshly isolated unwashed MAs in the electron microscope. Thus we conclude that sucrose has no immediate effect on the structure of the MA.

pH 6.1 and 6.4 MAs were fixed for electron microscopy after 1 week in sucrose. No differences between these MAs appear in the electron microscope. Regions containing microtubule and pieces of microtubules were found (Fig. 17), but in some areas there was a breakdown of microtubule structure. In the latter areas, linear arrays of ribosome-like particles remained intact.

The data for several of these experiments are found in Table 6. In all experiments there was a significant decrease in retardation following the washing. At pH 6.1 the washed MAs maintained their structure fairly well when observed with polarized light and phase-contrast microscopy, although some of them appeared slightly swollen. A few of these MAs lost their asters (see, for example, Fig. 18), but only MAs with asters were used for the measurements. Washed pH 6.2 and 6.3 MAs were identical in morphology to washed pH 6.1 MAs.

Washed MAs isolated at pH 6.1–6.3 appeared similar when viewed with the electron microscope. Microtubules were rather difficult to find in these MAs, and when found they had certain morphological differences from those found in unwashed MAs. The

<table>
<thead>
<tr>
<th>pH of isolation medium</th>
<th>No. of measurements</th>
<th>Untreated (no sucrose)</th>
<th>Treated with sucrose and returned to fresh medium</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1</td>
<td>10</td>
<td>39</td>
<td>34</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>6.4</td>
<td>10</td>
<td>36</td>
<td>35</td>
<td>&lt; 0.06</td>
</tr>
</tbody>
</table>
walls were no longer as thick or as smooth as those seen in unwashed MAs, and many more kinks were present (Fig. 19). All of these conditions can lead to a decrease in the measured retardation, as they would affect the amount and degree of orientation of the oriented components of the MA.

All of the washed MAs isolated at pH 6.4 and examined with the electron microscope appeared as in Fig. 20. This electron micrograph shows a chromosome-to-pole region from an anastral MA such as the one depicted in Fig. 18. No distinct microtubules were seen, but short segments of fibres and particles were detected. The chromosomes appeared swollen and less dense than in unwashed MAs. Washed pH 6.4 MAs may have contained microtubules, but it is apparent that the number of these microtubules must be quite small, since none were observed in the many thin sections examined with the electron microscope.

<table>
<thead>
<tr>
<th>pH of isolation medium</th>
<th>No. of measurements</th>
<th>Unwashed MAs</th>
<th>Washed MAs</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1</td>
<td>10</td>
<td>43</td>
<td>28</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>6.1</td>
<td>10</td>
<td>49</td>
<td>39</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>6.4</td>
<td>10</td>
<td>38</td>
<td>27</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>6.4</td>
<td>10</td>
<td>39</td>
<td>30</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>6.4</td>
<td>10</td>
<td>38</td>
<td>30</td>
<td>&lt;0.02</td>
</tr>
</tbody>
</table>

Table 7. 2M sucrose wash experiment
(MAs isolated at pH 6.3.)

<table>
<thead>
<tr>
<th>No. of measurements</th>
<th>Unwashed</th>
<th>Washed in sucrose 10 times</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>49</td>
<td>47</td>
<td>&lt;0.4</td>
</tr>
</tbody>
</table>

Stabilization of MA structure during the washing procedure

Because sucrose slows the deterioration of MA structure during storage, it was decided to wash MAs in the presence of sucrose in an attempt to prevent the damage caused by the normal washing procedure. It was found that sucrose preserved MA structure even after 10–15 washings. Table 7 contains the birefringence data for an experiment in which the MAs were washed 10 times in cold isolation medium containing 2 M sucrose. After washing, the MAs were resuspended in the normal hexylene glycol medium and measured immediately with the polarization microscope. There was no significant difference in retardation after washing with sucrose, and the ultrastructural morphology appeared identical to that of the unwashed MA.

The gel-like nature of the MA (see Heilbrunn, 1943; Mazia, 1961; Cohen, 1966) has been discussed for many years. Since divalent cations are important for the
stabilization of some gels, repeated washing in the divalent cation-free isolation medium might result in the loss of structural integrity of the MA. It was therefore decided to investigate the effects of divalent cations on MA stability.

When 0.003 M MgCl₂, MgSO₄, CaCl₂, or CaSO₄ were added to the wash medium (the effects of the latter three salts were tested on MAs from *Echinus esculentus*, kindly supplied by the Scottish Marine Biological Association, Millport) the MAs retained their birefringence, and in some cases the retardation increased after washing. Table 8 contains the data for experiments at pH 6.1 and 6.4, utilizing 0.003 M MgCl₂ in the wash medium. The other Mg²⁺ and Ca²⁺ salts gave identical results. The increase in retardation could be due to the washing away of some of the amorphous gelled material surrounding the MA, thus increasing the refractive index difference between the form birefringent components of the spindle and its background. The microtubules in MAs washed with Mg²⁺ or Ca²⁺ salts were identical in appearance to those in unwashed MAs (Fig. 21), regardless of the pH of the isolation medium. To make sure that the washing effect was due to the divalent cations and not to the anions (e.g. Cl⁻), similar concentrations of NaCl and KCl were added to the wash medium. In both cases the normal decrease in retardation was found after washing. This also demonstrates that Na⁺ and K⁺ do not prevent the loss of birefringence with washing.

Table 8. Magnesium-washed MAs
(MAs washed 3 times with 0.003 M MgCl₂ in isolation medium.)

<table>
<thead>
<tr>
<th>pH of isolation medium</th>
<th>No. of measurements</th>
<th>Average retardation values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Unwashed MAs</td>
</tr>
<tr>
<td>6.1</td>
<td>10</td>
<td>36</td>
</tr>
<tr>
<td>6.1</td>
<td>10</td>
<td>45</td>
</tr>
<tr>
<td>6.4</td>
<td>10</td>
<td>32</td>
</tr>
<tr>
<td>6.4</td>
<td>10</td>
<td>28</td>
</tr>
</tbody>
</table>

It is concluded that magnesium or other divalent cations such as calcium are involved in preserving the structure of the isolated MA. In support of this Mazia (1959) has reported that 2 × 10⁻⁴ M Ca²⁺ 'stabilized' MAs isolated with dithiodiglycol. Kane (1962a) reported that disintegration of isolated MAs at high pH was prevented by the addition of 10⁻³ M Ca²⁺ or Mg²⁺, and Sisken et al. (1967) found it necessary to include divalent cation in the medium used for isolating MAs from cells in culture. Furthermore, in some cells, low concentrations of divalent cations are necessary to preserve spindle microtubules fixed at relatively high pH for electron microscopy (Roth & Daniels, 1962; Harris, 1962).

Other properties of MAs treated with Mg²⁺

MAs may be isolated in the presence of 0.003 M MgCl₂, although the resulting preparation contains large clumps of cellular debris. It was of interest to determine whether the presence of Mg²⁺ in the isolation medium resulted in a change in retarda-
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Isolation of freshly isolated MAs. Eggs from a single sea urchin were divided in half: one half for isolation with Mg\(^{2+}\) and the other half for isolation without Mg\(^{2+}\). Table 9 provides the data which demonstrate a significant increase in retardation when Mg\(^{2+}\) is included in the isolation medium, at either pH 6.1 or 6.4.

The long-term effects of Mg\(^{2+}\) were also investigated. Tables 10 and 11 show that pH 6.1 and 6.4 MAs isolated with (Table 11) or without (Table 10) Mg\(^{2+}\) in the original isolation medium sustained their original retardations after 48 h. These MAs were stored (0-4 °C) in medium containing Mg\(^{2+}\), after having been washed three times in isolation medium containing Mg\(^{2+}\).

Table 9. Magnesium effect on retardation of unwashed MAs
(MAs isolated with and without Mg\(^{2+}\) in isolation medium.)

<table>
<thead>
<tr>
<th>pH of isolation medium</th>
<th>No. of measurements</th>
<th>Average retardation values</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Unwashed without Mg(^{2+})</td>
<td>Unwashed with Mg(^{2+})</td>
</tr>
<tr>
<td>pH of isolation medium</td>
<td>No. of measurements</td>
<td>Unwashed without Mg(^{2+})</td>
<td>Unwashed with Mg(^{2+})</td>
</tr>
<tr>
<td>6.1</td>
<td>10</td>
<td>32</td>
<td>41</td>
</tr>
<tr>
<td>6.4</td>
<td>10</td>
<td>22</td>
<td>37</td>
</tr>
</tbody>
</table>

Table 10. Preservation of birefringence after isolation without Mg\(^{2+}\), followed by three washes with Mg\(^{2+}\)

<table>
<thead>
<tr>
<th>pH of isolation medium</th>
<th>No. of measurements</th>
<th>Average retardation values</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0–30 min</td>
<td>48 h later</td>
</tr>
<tr>
<td>pH of isolation medium</td>
<td>No. of measurements</td>
<td>Unwashed without Mg(^{2+})</td>
<td>Unwashed with Mg(^{2+})</td>
</tr>
<tr>
<td>6.1</td>
<td>10</td>
<td>30</td>
<td>27</td>
</tr>
<tr>
<td>6.4</td>
<td>10</td>
<td>23</td>
<td>21</td>
</tr>
</tbody>
</table>

Table 11. Preservation of birefringence after isolation with Mg\(^{2+}\) and three washes with medium containing Mg\(^{2+}\)

<table>
<thead>
<tr>
<th>pH of isolation medium</th>
<th>No. of measurements</th>
<th>Average retardation values</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0–30 min</td>
<td>48 h washed</td>
</tr>
<tr>
<td>pH of isolation medium</td>
<td>No. of measurements</td>
<td>Unwashed without Mg(^{2+})</td>
<td>Unwashed with Mg(^{2+})</td>
</tr>
<tr>
<td>6.1</td>
<td>10</td>
<td>41</td>
<td>48</td>
</tr>
<tr>
<td>6.4</td>
<td>10</td>
<td>37</td>
<td>36</td>
</tr>
</tbody>
</table>

Increase in retardation after fixation

MAs, isolated without Mg\(^{2+}\) at the various pHs and then fixed, were examined with polarized light and with phase contrast to determine whether any structural changes take place after fixation. With phase contrast the MAs appeared identical to the unfixed material. This was also true in general for the fixed MAs observed in polarized light, however, there were a few MAs which appeared more fibrous than normal. In these, the chromosomal fibres seemed to stand out more against their background.
In almost all cases investigated, except for one experiment at pH 6.4, there was an increase in retardation following fixation of the MAs. This is in contrast to the results reported by Rebhun & Sander (1967) using similar material. No reason for this discrepancy could be found.

Cytochemical analysis of the isolated MA ribonucleic acid.

Cytochemical methods were employed in an effort to determine whether the particles present in MAs are ribosomes. Freshly isolated MAs were fixed, without washing, in 2.5% glutaraldehyde at pH 6.1-6.4. For light microscope identification of RNA in the MA, the azure B bromide technique (Flax & Himes, 1952) was employed. The MAs stained intensely purple, which is a positive test for RNA. RNase treatment for 1-1 h at room temperature (see Materials and Methods) resulted in a complete loss of this staining, which is also characteristic for RNA.

After 1-2 h of RNase treatment at room temperature the only fine-structural components altered in RNase treated MAs were the particles normally localized along the microtubules and in inter-microtubular regions (Fig. 22). Granular material remained deposited on the microtubule surfaces, but this material appeared to be more amorphous and less distinctly spherical than the ribosome-like particles normally seen distributed around the microtubules. Inter-microtubular ribosome-like particles seemed to be completely missing in most of the sections observed. Control MAs for the RNase reaction, which had been placed in distilled water for equivalent lengths of time, retained normal particle distribution and morphology.

DISCUSSION

The results described above lead us to certain insights into the stability and the nature of the birefringent material in the isolated sea-urchin MA. They clearly indicate the sensitivity of the birefringence of the MA to slight changes in the pH of the isolating medium; changes which may reflect similar responses of the MA within the living cell. Furthermore, the decrease of birefringence and the disappearance of microtubules with storage and with repeated washing suggest that a factor necessary for the stability of these components is washed or leached out of the MA under these conditions. These changes can be prevented by sucrose and by the divalent cations calcium and magnesium. Although sucrose can hardly be considered a likely candidate for the maintainance of the MA in vivo, it is reasonable to assume that divalent cations might be involved, as has been suggested by Roth & Daniels (1962) and by Harris (1962). The in vitro studies of the stability of the birefringent component of the MA raise the possibility that regulation of hydrogen ion and/or divalent cations may set the stage for the appearance or disappearance of the birefringent components of the MA.

Some controversy has arisen as to the source of birefringence of the MA, i.e. as to the identity of the ultrastructural entities which give rise to this light-microscope property of the MA. Behnke & Forer (1966) have argued against the hypothesis that the majority of the birefringence is due to microtubules. They base their conclusions on combined polarized light and electron microscopy of Nephrotoma spermatocyte
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MAs. These MAs contain chromosomal fibres which measure 1 μ in thickness when observed with polarized light, but no bundles of microtubules corresponding to the fibres are seen when observed with the electron microscope. They also point out that in polarized light, chromosomal fibres are seen to end on the chromosomes, but no terminations of microtubules at the chromosomes are seen in their electron-microscope preparations. Most of the microtubules pass through the chromosomes and are therefore not considered by them to be correlated with the birefringent fibres. Bundles of tightly packed microtubules, and microtubules ending on chromosomes are observed in the isolated MA in the present study. These most likely correspond to the chromosomal fibres seen in the isolated MA with polarized light.

The fact that birefringent fibres are not seen passing through the chromosomes in Behnke and Forer's observations may originate from a high concentration of material in the chromosomes which may closely match the refractive index of the microtubules, and thus cancel some of the form birefringence of the latter (Rebhun & Sander, 1967).

Lastly, Behnke & Forer (1966) point out that there is no birefringence in the interzonal region at anaphase and yet there are many microtubules in the interzonal region. The several possible explanations for this apparent lack of birefringence have been considered in detail by Rebhun & Sander (1967). Furthermore, isolated anaphase MAs of sea urchins do have a low level of birefringence in the interzone, which also contains microtubules.

These arguments support the idea that, qualitatively, birefringence in the isolated MA in part arises from microtubules. If we add to these considerations the extensive correlation of parallel decrease in retardation with the disappearance of microtubules during storage (see also Kane & Forer, 1965) and during washing, and conversely the preservation of both birefringence and microtubules with divalent cation or sucrose, we obtain further support for the qualitative correlation of the birefringence of the isolated MA with oriented microtubules. A similar correlation has been pointed out in other material by Rebhun & Sander (1967).

Birefringence has also been correlated with microtubules in several other instances. For example, Tilney (1965) found that the birefringence of Heliozoan axopods was associated with the presence of microtubules. Taylor (1966) found microtubules in the microspikes of spread tissue culture cells, and Goldman (unpublished results) demonstrated that these microspikes are birefringent.

We have stressed the qualitative statement that birefringence can originate from oriented microtubules and not that all birefringence necessarily originates from this source, for the simple reason that our evidence suggests otherwise. We refer to the experiments described above in which microtubules as such disappear from aged MAs, although not all the birefringence disappears. The residual birefringence appears to be correlated with the linear aggregates of ribosome-like particles held together by an ill-defined amorphous material, which may be a breakdown product of the microtubules. That such linear aggregates should give rise to form birefringence appears clear from theories of birefringence (Bennett, 1950), especially the recent analysis put forth by Cassim & Taylor (1965). In fact, the ribosome-like particle aggregates reported here seem like an almost ideal physical correlate to the beaded chain model...
there analysed. The greater residual birefringence in MAs isolated at pH 6.1 (after 48–72 h), as compared with those isolated at pH 6.4 (after 24 h) emphasizes this correlation, since at pH 6.4 the particles are considerably more dispersed, and less well oriented.

It appears clear therefore that in the isolated sea-urchin MA birefringence can arise from oriented material other than microtubules, although such material may owe its orientation to its association with microtubules. It is not known whether microtubule-ribosome aggregates occur in living eggs (they do not appear in most fixed whole eggs containing MAs according to the electron micrographs of Harris, 1962) or whether they are an artefact of our isolation procedure. Thus it is questionable whether such a phenomenon must be evaluated in understanding birefringence in living cells. However, the possibility that these considerations are not totally irrelevant to living cells comes from the recent work of Forer (1966).

In 1966 Forer provided evidence that the in vivo MA contains more than one oriented component involved in chromosome movement by irradiating living cells with an ultraviolet microbeam. Two of the radiation effects observed were: (1) the movement of chromosomes when chromosomal fibre birefringence was reduced to a low level by the u.v. microbeam, and (2) the lack of anaphase chromosome movement after u.v. irradiation, even though the birefringence of chromosomal fibres was maintained. From Forer's experiments it seems that the component providing the low level of birefringence may be important in moving the chromosomes and thus, if the in vivo material showing low birefringence corresponds to that providing birefringence after the microtubules of the isolated MA disappear, the particles may, in some manner, be involved in chromosome movement. In such a scheme the microtubules are considered to be a cytoskeletal system, needed for support and for guiding the chromosomes towards the poles, rather than for the generation of motive power. It must be emphasized that the relation of Forer's (1966) results to our own observations on isolated MAs is highly speculative.

A detailed analysis of the physico-chemical basis for the association of particles and microtubules is impossible at the present time. However, since some of the particles are likely to be ribosomes (on the basis of our cytochemical experiments and those experiments of Hartman & Zimmerman (1968), who have isolated 74s ribosomes from isolated MAs), in vitro studies of ribosomes suggest some interesting possibilities. Several investigations have shown that, in vitro, ribosomes bind a wide variety of proteins (Siekevitz & Palade, 1960, 1962; Schwartz & Petermann, 1966). According to Schwartz & Petermann (1966), proteins bound to ribosomes may come from several sources, including newly synthesized protein which remains bound to ribosomes during isolation, proteins which are involved in protein synthesis, and most commonly, proteins non-specifically adsorbed during isolation. By analogy, it is suggested that the microtubule proteins bind to the ribosome-like particles forming the complex seen in the isolated MA. Since relatively small changes in pH result in large changes in ribosomal binding constants (Petermann & Hamilton, 1961) the differences in state of aggregation of ribosomes at pH 6.1 and 6.4 may be due to differences in binding capacity between ribosomes and microtubules in the isolated MA at the two pHs.
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Furthermore, MAs washed without Mg\textsuperscript{2+} retain normal particle structure as far as can be observed with the electron microscope. This is not to be expected if the particles are ribosomes, as ribosomes are known to break down to smaller subunits in the absence of magnesium, and 2–3 washes in fresh isolation medium are likely to remove most of the divalent cation which may be present. However, Schwartz & Petermann (1966) point out that ribosomal particles can be preserved in the absence of magnesium by the addition of a protein such as cytochrome, and with higher concentrations of cytochrome, dimers and trimers of ribosomes are formed (Schwartz & Petermann, 1966). In the present case, non-specifically or specifically adsorbed protein, derived either from microtubules or from other cytoplasmic proteins may be responsible for the preservation of ribosome-like particles in the washed and, presumably, divalent cation-free MA.

The morphological observations in this study may be of interest in the interpretation of the most recent work on isolated MA proteins (Kane, 1967; Stephens, 1967; Sakai, 1966). Kane (1967) and Stephens (1967) used washed, hexylene glycol MAs isolated at pH 6.4 for their study of the major MA protein. The washed MAs used by Kane and Stephens disintegrated in 0.6M KCl when observed with the phase-contrast microscope. The major soluble component of their disintegrated MAs was a protein which sedimented at 22s in the ultracentrifuge. In addition, there was a small heterogeneous 4–5s component. Both Kane and Stephens considered the 22s material to be microtubule protein.

Our experiments with polarized light and electron microscopy have shown that washed pH 6.4 MAs show ultrastructural deterioration and loss of microtubules. The 'fibres' seen with the phase-contrast microscope in washed pH 6.4 MAs (Kane, 1967) are not necessarily composed of microtubules. They may correspond with the short chains of particles seen in our washed pH 6.4 MAs, or perhaps to material not visible in electron-microscope preparations. It therefore seems likely that Kane (1967) and Stephens (1967) have isolated a protein which could be involved in the structure of the MA, but which is not necessarily identical with the microtubule protein.

It is clear from our electron-microscope preparations that microtubules make up only a small proportion of the volume of the isolated MA. The majority of the MA structure is made up of vesicles, ribosome-like particles, and 'empty spaces' separating the various structures. These 'spaces' may contain an amorphous material, perhaps a protein, which could act as a matrix for holding the constituents of the isolated MA together. Thus Kane (1967) and Stephens (1967) could be dealing with a protein derived from several different sources. Further support for this conclusion is found in the work of Borisy & Taylor (1967), who demonstrated that the 22s protein of Kane is not the colchicine-binding protein thought to be derived from microtubules.

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REFERENCES


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(Received 5 April 1968)

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Fig. 1. Unwashed metaphase MA observed with phase-contrast optics. Note the chromosomes (chr) at the metaphase plate, asters (ast), and the spindle fibres (spf).

Fig. 2. Unwashed MA observed with Nomarski differential interference optics. Note chromosomes (chr), spindle fibres (spf), and astral regions (ast).

Figs. 3, 4. Unwashed MAs observed with polarized light at opposite compensator settings. Note the chromosomes (chr), birefringent spindle region, chromosomal fibres (spf), and the 'maltese cross' in the astral region (ast). The 'rim' of isotropic cytoplasm (ic) is evident in Fig. 3.

Fig. 5. Longitudinal section through a whole spindle region of an MA isolated at pH 6.2. Most of the particles (p) are associated with the fibres (f). Chromosomal fibres (chf), continuous fibres (cf), and pieces of fibres in the chromosome-to-pole region are seen. Chromosomes and vesicles are also seen. Fixed in 1% osmium tetroxide. (chr, chromosomes.) × 6200.
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Fig. 6. Chromosome-to-pole region of pH 6:1 unwashed MA. Bundles of microtubules (bt) and single microtubules (t) are present. Ribosome-like particles are on the surface of the microtubules (p), and seem to be linked by a less dense amorphous material (f). 'Gracefully bending' (gbt) and 'straight' (st) microtubules are seen. Polysome-like configurations (poly) are located in the spaces between the microtubules, as are the vesicles (v). Fixed in 1 % OsO₄. x 50,000.

Fig. 7. Chromosome-to-pole region of pH 6:1 unwashed MA. Note that some regions of the microtubules are free of particles and others are densely packed with the particles. Aggregates of particles appear to interconnect some microtubules. Note the large bundle of microtubules (bt) and the 'gracefully bending' tubule (gbt). Kinked microtubules are also present (kt). Fixed in 1 % OsO₄. x 23,000.

Fig. 8. A higher magnification electron micrograph of the bundle of microtubules (bt) seen in Fig. 7. This bundle consists of eight microtubules. Note the amorphous material (f) associated with the ribosome-like particles (p), and the interconnecting aggregates of particles. x 65,000.

Fig. 9. Cross-section through the chromosome-to-pole region of a pH 6:1 unwashed MA. Cross-sections (xs) of single microtubules, doublets, and larger bundles are present. Fixed in 1 % OsO₄. x 37,700.
Fig. 10. Longitudinal section through the chromosomal region of an unwashed pH 6-2 MA. Note the chromosomes (chr) and microtubules running directly towards the chromosomes (chrt). One microtubule is seen to end at the surface of the chromosome (e). Other microtubules are seen to pass between chromosomes, and probably represent elements of continuous fibres (ct). Ribosome-like particles are seen at the periphery of the chromosomes, and in some instances appear to be associated with the particles associated with nearby microtubules (see arrows). Fixed in 1% OsO₄. × 25000.

Fig. 11. Longitudinal section through a centriole (ce). Microtubules are seen in both cross (xt) and longitudinal (lt) sections in the surrounding astral region. pH 6-2 unwashed MA. Fixed in 1% OsO₄. × 21000.

Fig. 12. Chromosome-to-pole region of an unwashed pH 6-4 MA. The ribosome-like particles (p) seem to be more loosely associated with the microtubules (t). Fixed in 2.5% glutaraldehyde. × 28600.

Fig. 13. A pH 6-1 unwashed MA stored at 0-4 °C for 72 h and then observed in polarized light. Birefringence is detectable and retardation values can be measured.
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Fig. 14. Chromosomal region of a pH 6·4 MA stored in the lysate for 20 h (0–4 °C). Note the lack of orientation and the random distribution of particles (p). Sometimes there are slight indications of oriented particles (see arrows); however, no microtubules are present. Many of the particles appear to be associated with amorphous material (f). Fixed in 1% OsO₄. ×42000.

Figs. 15, 16. Longitudinal sections through the chromosome-to-pole region of MAs isolated at pH 6·1 and stored in the lysate (0–4 °C) for 72 h. Microtubules are no longer present, but the linear arrays of particles remain in place (p). The chromosomes appear to be quite dense (chr). Amorphous electron-dense material (md), may represent microtubule breakdown products. Fixed in 1% OsO₄. Fig. 15, ×25000. Fig. 16, ×28000.
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Fig. 16. See p. 204.

Fig. 17. Longitudinal section through the chromosome-to-pole region of a pH 6·1 MA stored in 2 m sucrose for 1 week. Note the presence of microtubules (see arrows). Fixed in 2·5 % glutaraldehyde. × 50000.

Fig. 18. Washed pH 6·4 MA, viewed with the polarization microscope immediately after washing. Note that the asters are missing.

Fig. 19. Longitudinal section of a washed pH 6·1 MA. The microtubule walls (w) appear to be fragile and are not as thick or as smooth as those seen in the unwashed MA preparations. Many of the microtubules are kinked (k). Fixed in 1 % OsO4, × 31100.
Isolated mitotic apparatus
Fig. 20. Longitudinal section through the chromosome-to-pole region of a pH 6.4-washed MA. No distinct microtubules are present. Short ribosome-like particle associations are seen intertwined with amorphous material (fp). The chromosomes (chr) appear swollen and less dense. Fixed in 1% OsO₄. x 15800.

Fig. 21. Longitudinal section through the chromosome-to-pole region of an MA isolated at pH 6.4 and washed with isolation medium containing 0.003 M Mg²⁺. Fixed in 1% OsO₄. x 35000.

Fig. 22. Longitudinal section of an RNase-treated unwashed MA isolated at pH 6.3. Most of the ribosome-like particles have disappeared; however, some granular material (g) remains deposited on the microtubule walls. Fixed in 2.5% glutaraldehyde. x 29700.
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