ACTION OF COLCHICINE AND HEAVY WATER ON THE POLYMERIZATION OF MICRO-TUBULES IN WHEAT ROOT MERISTEM

J. BURGESS AND D. H. NORTHCOTE
Department of Biochemistry, University of Cambridge, England

SUMMARY
The effects of colchicine and heavy water on cell division in wheat roots have been examined by means of mitotic index measurements correlated with an electron-microscope examination of the tissue. Heavy water alone caused an arrest of mitosis both in normal tissue and in roots which had been brought into partial synchrony of division by means of 5-aminouracil. Colchicine alone caused an accumulation of cells at c-metaphase in the synchronized tissue. Colchicine dissolved in heavy water showed variable effects; at high heavy water concentrations (greater than 70 %) mitosis was arrested; when the heavy water concentration was less than 70 %, the mitotic index was approximately equal to that obtained by treatment of the tissue with colchicine dissolved in ordinary water.

These actions of colchicine and heavy water on the mitosis of the cells have been related to the formation and organization of the microtubules in the dividing cells. Heavy-water treatment was characterized by an increase in the number of microtubules found in the preprophase band and in the mitotic spindle. The microtubules of the preprophase band in cells treated with heavy water were arranged in a highly disoriented manner. After colchicine treatment the numbers of microtubules at the preprophase band and in the mitotic spindle were depleted. The preprophase band consisted of a few microtubules situated close to the wall. In the mitotic spindle the microtubules were either not present or visible only in the vicinity of profiles of endoplasmic reticulum or the chromosomes. When the cells were exposed to a solution of colchicine in 70 % heavy water microtubules were absent from the mitotic spindle and the preprophase band consisted of a few microtubules which were sometimes found scattered both along the wall and dispersed further into the cytoplasm.

INTRODUCTION
Colchicine is the best known and most extensively studied inhibitor of spindle function. It induces a modified type of mitosis—termed c-mitosis by Levan (1938)—characterized principally by a long delay in the separation of the daughter chromosomes, leading to the condition of c-metaphase. Molé-Bajer (1958) has published a detailed account of c-mitosis as recorded by cinematography. He states that during prophase, chromosome movements are due to intrinsic factors within the chromosomes themselves, rather than to any interaction between the spindle and the centromeres. Inoué (1952) has shown that colchicine causes the disappearance of the spindle birefringence, and that the effect is reversible. The possible correlation between spindle birefringence and the organization of the spindle tubules (Harris & Bajer, 1965) suggests that colchicine may have a direct effect on the ultrastructural organization of the spindle, and particularly on the microtubules. Ultrastructural studies have consistently shown that
colchicine treatment reduces the number of microtubules present in the cell (Robbins & Gonatas, 1964; Behnke, 1965; Pickett-Heaps, 1967). Heavy water is known to inhibit growth in roots (Pratt & Curry, 1937) and to arrest mitosis in cleaving sea-urchin eggs (Gross & Spindel, 1960). It appears to act by overstabilizing the gel structure of the spindle (Marsland & Zimmermann, 1963), and its effects on these eggs may be partially overcome by raising the hydrostatic pressure on the cells (Marsland, 1965) or by lowering the temperature of the medium (Marsland & Asterita, 1966). High hydrostatic pressure and low temperature are known to affect the stability of microtubules in a variety of tissues (Tilney, Hiramoto & Marsland, 1966; Behnke, 1967; Roth, 1967; Goode, 1967; Tilney & Porter, 1967). The ultrastructural effects of exposing cells to heavy water have not been examined.

The work to be described is a study of the action of colchicine and heavy water on the cells of wheat roots. The effects have been examined with the electron microscope and by means of mitotic index measurements on fixed tissue. A partially synchronized cell population (Clowes, 1965; Burgess & Northcote, 1967) has been used in some of the experiments.

MATERIALS AND METHODS

Root tips intended for optical microscopy were fixed in methanol–chloroform–propionic acid fixative (6:3:2, v/v) for 16 h at 4 °C. They were then dehydrated in Cellosolve and embedded in Paraplast medium using standard techniques. Sections (8 μm) were cut on a Jung rotary microtome, mounted on gelatin-coated glass slides and stained with Harris' haematoxylin. Mitotic index counts of the root tip were made using a Zeiss Ultraphot II microscope. Approximately 1000 cells were examined in each of 3 root tips for each experimental point. The material to be examined in the electron microscope was fixed in buffered 6 % glutaraldehyde solution containing salts for 1 h at room temperature (Burgess & Northcote, 1967). It was then washed, post-fixed in buffered 1 % osmium tetroxide for 1 h at room temperature, dehydrated in a graded aqueous ethanol series, and embedded in Araldite.

Cells in the root meristem were brought into partial synchrony of division by means of a 24-h exposure to a solution of 5-aminouracil (Clowes, 1965).

EXPERIMENTAL AND RESULTS

Mitotic-index measurements

Effect of synchronization procedure. Roots were immersed in a solution of 5-aminouracil (4 mM) for 24 h in the dark. They were then removed to aerated water, and sampled at hourly intervals for 14 h. Figure 1 is a graph showing the variation of mitotic index with time after removal to tap water.

Effect of colchicine after synchronization. At various times after removal of the roots to aerated water, samples were transferred to an aerated 0.1 % (w/v) solution of colchicine. Roots were fixed 11 h after their removal from the 5-aminouracil solution. This corresponded to the peak of mitosis in roots treated with 5-aminouracil alone (Fig. 1). In a partially synchronized cell population of this type, colchicine would be expected to hold in mitotic configuration all those cells entering mitosis during the period of treatment, provided that this time does not exceed that for formation of a single restitution nucleus. The results of this experimental series are given in Table 1.
Polymerization of microtubules

**Effect of heavy water (D₂O) without synchronization.** Roots of whole seedlings were immersed in heavy water (> 99%, Koch-Light Ltd.). Samples were fixed at hourly intervals. After 3 h the mitotic index in the treated roots had fallen to 0.5%, or less, and remained low. If, after treatment for 5 h, roots were transferred to water (H₂O), a partial recovery occurred over the next 3-h period. The extent of this recovery was variable (Fig. 2).

![Graph of mitotic index variation](image)

**Fig. 1.** Variation of mitotic index in wheat root tips with time after a 24-h treatment with 5-aminouracil (4 mM).

<table>
<thead>
<tr>
<th>Period of recovery before transfer to colchicine (h)</th>
<th>Time in colchicine (h)</th>
<th>Mitotic index at 11 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>4</td>
<td>50–60%</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>30–35%</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>25–35%</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>25–35%</td>
</tr>
</tbody>
</table>

**Effect of heavy water after synchronization.** Two types of experiment were performed. In the first the roots, after a certain time in aerated water, were transferred to pure (> 99%) heavy water and sampled at intervals. If the roots were placed in the heavy water at any time up to and including 8 h after washing away the 5-aminouracil, the peak of mitosis at around 11 h was abolished. If the roots were exposed to the heavy water 9 or 10 h after washing away the 5-aminouracil, the peak at 11 h was present, although diminished. Regardless of the timing of the transfer to heavy water, roots which had been immersed in heavy water for more than 3 h always showed a mitotic index of 0.5% or less (Fig. 3).
In the second series of experiments, roots were transferred 8 h after removal from the 5-aminouracil solution into solutions of D$_2$O in H$_2$O. The concentrations of heavy water were 50%, 70%, and 90% (v/v). Three hours later—that is, at the expected time of the peak of mitosis—samples were fixed and examined. The results are given in Table 2. If roots were sampled between 3 and 8 h after transfer into the heavy water.
water solutions they showed no peak of mitosis. The effect of the heavy water thus seems to be an inhibition (arrest) of mitosis rather than a delay in time.

**Effect of simultaneous exposure to heavy water and colchicine.** Eight hours after removal of the roots from 5-aminouracil, samples were placed in a 0.1% (w/v) solution of colchicine in 50%, 70%, 90% and > 99% (v/v) D₂O in H₂O. Three hours later samples were fixed. The results are given in Table 2.

<table>
<thead>
<tr>
<th>Concentration of D₂O in H₂O</th>
<th>Last 3 h of recovery in:</th>
<th>Colchicine (0.1%) in D₂O/H₂O mixtures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D₂O/H₂O alone</td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>25-30% (control)</td>
<td>30-35%</td>
</tr>
<tr>
<td>50%</td>
<td>7-10%</td>
<td>13-15%</td>
</tr>
<tr>
<td>70%</td>
<td>5-8%</td>
<td>9-12%</td>
</tr>
<tr>
<td>90%</td>
<td>0.5-1%</td>
<td>0.5-1%</td>
</tr>
<tr>
<td>99%</td>
<td>0.5-1%</td>
<td>0.5-1%</td>
</tr>
</tbody>
</table>

**Electron microscopy**

**Colchicine.** Roots were exposed to 5-aminouracil solution for 24 h and allowed to recover in aerated water for 9.5 h. They were then transferred to a 0.1% (w/v) solution of colchicine and fixed 90 min later. This treatment allows the examination of large numbers of cells in the active stages of mitosis.

The distribution of microtubules in normally growing root tissue has been described before (Pickett-Heaps & Northcote, 1966; Burgess & Northcote, 1967). After colchicine treatment the randomly distributed wall microtubules of interphase were usually still present, although it was possible to find cells in which no microtubules were visible. It is difficult to make an assessment of the effect of the colchicine treatment on this population of microtubules since they are visible in variable numbers even in normally growing tissue.

At preprophase an examination of the equatorial regions of the wall showed that the preprophase band of microtubules was present, but that it consisted of a comparatively depleted number of microtubules. In normally growing tissue, the most common configuration of the band contains microtubules 4-6 deep (Pickett-Heaps & Northcote, 1966). In a corresponding band after colchicine treatment the microtubules lie only 1-2 deep along the equatorial region of the wall. The lateral extension of the band is comparable in both cases, and microtubules are absent from the rest of the wall. Occasionally it is possible to see other organelles associated with the microtubules. A depleted band, together with a large number of darkly staining circular vesicles and a few profiles of smooth endoplasmic reticulum, is shown in Fig. 4. The association of these two types of organelle with microtubules has been noted previously (Burgess & Northcote, 1968).

An examination of dividing cells after colchicine treatment showed that typically the
chromosomes were distributed randomly throughout the spindle area. The area between
the chromosomes contained ribosomes, vesicles and endoplasmic reticulum, and
occasionally Golgi bodies. Microtubules were either absent or, if present, visible
only for short distances from their point of attachment to chromosomes (Fig. 5), or as
short pieces in the nucleoplasm, often in the vicinity of profiles of endoplasmic reticulum.

Cell-plate formation appeared not to proceed normally after treatment of the cells
with colchicine solution. Comparatively mature plates could be seen to end abruptly
in the cytoplasm and examination of the edges showed that only a few microtubules
were present (Fig. 6).

Heavy water. Since heavy water abolishes the effect of the synchronization procedure,
roots were simply placed in pure (> 99%) heavy water for 5 h before fixation. This
treatment gave a low incidence of mitotically active cells, but was not so drastic that the
tissue did not recover if transferred to water (H₂O) (see Fig. 2).

The interphase-wall microtubules showed no apparent response to the treatment.
However, the appearance of the preprophase band of microtubules was strikingly
different. It consisted of a very large number of microtubules lying up to 15 deep in
the cytoplasm. Moreover, this large number of microtubules showed a considerable
degree of disorganization, so that individual sections contained microtubules in many
different orientations. The disorientation sometimes appeared only at the cytoplasmic
edge of the band (Fig. 7), whilst in other cases it extended almost to the wall (Fig. 8).
Occasionally profiles of smooth endoplasmic reticulum could be seen among the
microtubules (Fig. 8).

The spindle area in mitotic cells after exposure to heavy water contained all the
usual components: chromosomes, endoplasmic reticulum, ribosomes and micro-
tubules. Often the area between the chromosomes was packed with large numbers of
microtubules (Fig. 9). At the edge of the spindle it was possible to follow single
microtubules for large distances in the section. Both these observations are in direct
contrast to the corresponding situations in colchicine-treated roots. Large numbers of
microtubules were also visible at the edges of the developing cell plate (Fig. 10).

Colchicine and heavy water. The effects of a single concentration of colchicine in
heavy water were investigated. Roots which had been allowed to recover from
5-aminouracil treatment for 8 h in aerated water were transferred to 70% (v/v) heavy
water containing 0.1% (w/v) colchicine. Three hours later they were fixed for electron
microscopy.

The pattern of interphase-wall microtubules was similar to that in cells exposed to
colchicine solution in water; some cells had an apparently normal population, whilst
others appeared to contain no wall microtubules at all.

The preprophase band appeared in two easily distinguishable forms. In some cells
it resembled the depleted form found after aqueous colchicine treatment—a few
microtubules lay 1–2 deep in the cytoplasm. In other cells the microtubules extended
quite far into the cytoplasm, but were still present in small numbers (Fig. 11). This
appearance of the band may be contrasted with the typical grouping of microtubules
after pure heavy water treatment; the microtubules extended for a similar distance into
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The cytoplasm, but after pure heavy water treatment they were packed closely together and were present in far larger numbers.

The mitotic spindle area contained Golgi bodies as well as the more normal organelles. Microtubules were usually absent, although short fragments were occasionally visible in the vicinity of endoplasmic reticulum. Cell-plate formation appeared to be inhibited, with plates ending abruptly in the cytoplasm, and having few or no microtubules at their edges. It thus appeared that in all the active stages of mitosis the effect of treatment with colchicine dissolved in heavy water was exactly analogous to the effect of colchicine in water.

DISCUSSION

A comparison between the action of colchicine and of heavy water on cell division in wheat roots has been made. In a partially synchronized cell population, the effects are opposite: colchicine causes an accumulation of cells at c-metaphase, leading to a high mitotic index, whilst heavy water causes a consistent drop in mitotic index to 0.5% or less after a 3-h treatment. In a randomly dividing cell population, such as a normally growing wheat root, heavy water again causes a depression in mitotic index after a lag of 3 h. The mitotic index in a randomly dividing cell population is a measure of the ratio of the time spent in mitosis to the intermitotic time. An increase in the time of mitosis leads generally to an increase in mitotic index, and the reverse is also true. It is therefore predictable from the fall in the mitotic index of the normally growing roots that heavy water either decreases the time of mitosis, or increases the intermitotic time, or both. Furthermore, the destruction of the peak of mitosis in a partially synchronized cell population suggests that the heavy water is capable of acting upon cells which are counted as interphase cells when viewed with the optical microscope; that is, it increases the intermitotic time. Conversely, the presence of an increased peak of mitosis in a partially synchronized cell population that has been treated with colchicine suggests that this agent has no drastic effect on cells in the stage before they can be seen by optical microscopy to have commenced mitosis.

Colchicine has been reported by several workers to cause depletion in the number of microtubules present in cells after fixation (Robbins & Gonatas, 1964; Behnke, 1965; Pickett-Heaps, 1967), whereas certain features of the inhibition of cleavage in sea-urchin eggs by heavy water suggest that microtubules may also be affected by this agent (Marsland, 1965; Marsland & Asterita, 1966).

The effect of colchicine and heavy water has been studied at each of the phases of cell division characterized by its own grouping of microtubules—interphase, preprophase, mitosis, and cell-plate formation (Burgess & Northcote, 1968).

In agreement with a similar study (Pickett-Heaps, 1967), it has been found that colchicine causes a partial disappearance of microtubules at all phases. In addition, smooth endoplasmic reticulum and darkly staining circular bodies, which are seen associated with microtubules in normally growing tissue (Burgess & Northcote, 1967, 1968), are visible in their expected positions after colchicine treatment. This implies that the colchicine may act directly on the material of the microtubules and not
indirectly by interfering with the transport mechanisms of the microtubule subunits (Burgess & Northcote, 1968). Direct binding of colchicine to a protein isolated from sea-urchin flagella has been demonstrated (Shelanski & Taylor, 1968).

Commenting upon the reduced number of microtubules which remained along the wall in xylem cells after treatment with 0.25% colchicine for 2–3 h, Pickett-Heaps (1967) suggested that the reason might be that the remaining microtubules had an especially sheltered environment. In the present study, residual microtubules have been found not only along the walls, but also in the spindle area in the vicinity of chromosomes and fragments of endoplasmic reticulum. Roth (1967) made a similar observation in cells of amoebae recovering from cold treatment. These residual microtubules presumably represent a more stable population. In the case of the residual microtubules in the mitotic spindle, this stability could be conferred by an association with the surface of either the chromosomes or the smooth endoplasmic reticulum. Borisy & Taylor (1967) have suggested that the kinetochore and centriole may be points for nucleation of microtubule growth. The residual microtubules of the preprophase band could again represent a more stable section of the whole population of microtubules at this phase. Nucleation of the mass of microtubules normally seen in the preprophase band could take place from this small number of stable units. The postulate of different stabilities amongst the microtubules in one cell is not unprecedented (Behnke & Forer, 1967).

Heavy water stabilizes the microtubule structures, leading to increased numbers of these organelles being visible in cells after fixation. The situation of preprophase is particularly interesting. The band consists of microtubules which are not only present in unusually large numbers, but which show a high degree of disorganization in terms of their relative orientation. In untreated cells the microtubules of the band are parallel, and sections showing a high degree of disorganization are only infrequently found (Burgess & Northcote, 1968). After heavy-water treatment the disorganized appearance is almost invariably seen. It may arise as a result of a displacement of the equilibrium between the microtubules and their constituent subunits. According to Marsland & Zimmermann (1965) this may be brought about by the displacement by D_2O of H_2O molecules present as a shell around the monomer binding sites before polymerization, and also by the stabilization of the polymers by substitution of D for H in the protein components of the system. This explanation of the action of heavy water has been proposed by Marsland & Zimmermann (1965), by analogy with the polymerization mechanism for tobacco mosaic virus protein (Ansevin & Lauffer, 1963). The presence of an agent that promotes polymerization of the microtubules may upset the normal sequence of aggregation which gives rise to an ordered grouping in the preprophase band, and could result in the disorganized appearance shown in Figs. 7, 8. In addition, it appears that in normally dividing cells the microtubule material in the preprophase band may be utilized in the formation of the mitotic spindle, since it has been shown that the preprophase band is present, and presumably dispersing, at a time when the microtubules of the mitotic spindle are already being formed (Burgess & Northcote, 1968). Stabilization of the microtubules of the preprophase band might thus be expected to have two effects: first, larger numbers of microtubules than usual
would be observed in the band, since mobilization to other parts of the cell would be inhibited; second, the onset of prophase might be arrested as a result of the absence of any spindle mechanism. The arrest of the onset of prophase would lead to inhibition of mitosis in a partially synchronized cell population. This has been observed to be the case. During mitosis in the presence of heavy water, the spindle is often crowded with large numbers of microtubules. These microtubules reflect the over-stabilization of the gel properties of the spindle observed in sea-urchin eggs (Marsland & Zimmermann, 1963).

An examination with the optical microscope of fixed materials after combined treatment of the roots with a solution of colchicine in heavy water showed that, apart from the variation in mitotic index tabulated in Table 2, roots treated with a solution of colchicine in 90% or 99% heavy water did contain cells in telophase configurations, whilst roots treated with more aqueous solutions (50%, 70% heavy water) did not. This implied that whereas 50% and 70% heavy water was ineffective in overcoming the metaphase block induced by the dissolved colchicine, 90% or 99% heavy water could overcome it. The single treatment examined in the electron microscope was 70% heavy water containing colchicine, and this would therefore be expected to show colchicine-like effects. This has been found true at all phases of cell division, except in the case of the type of preprophase band shown in Fig. 11. In a recent paper, Marsland & Hecht (1968) have examined the effects of a wide range of heavy-water solutions containing colchicine on the first cleavage in sea-urchin eggs. They found that over some concentration ranges the two substances were antagonistic, which is the result expected in terms of the proposed mechanism of action of the individual agents, but that over other concentration ranges the actions of colchicine and heavy water were synergistic. Whilst their study is in no way comparable to the work described here, it appears that simultaneous exposure of cells to heavy water and colchicine does not produce in every case the result which might be expected from current theories as to the action of these substances individually.

In summary, it is proposed that colchicine under the conditions described causes a partial loss of microtubules at all phases of mitosis. The remaining microtubules are presumed to represent a population whose stability either arises from the position of the microtubules in relation to other organelles, or is related to a functional specialization amongst the microtubules themselves. Heavy water causes an inhibition of mitosis in a partially synchronized cell population, and it is proposed that this effect is mediated through an arrest of the onset of prophase resulting from over-stabilization of the microtubules in the preprophase band. After heavy-water treatment the preprophase band contains a large number of microtubules in a state of disorganization. An examination of the effects of a solution of colchicine in heavy water has shown that most of the changes appear to result from a colchicine-like action of the mixed solution.

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REFERENCES


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ABBREVIATIONS ON PLATES

- c chromosome
- n nucleus
- cp cell plate
- t microtubules
- cw cell wall

Fig. 4. Cell treated with colchicine (0.1%) for 90 min during the 11-h recovery period of synchronization with 5-amino-uracil. The upper cell is at preprophase and the depleted preprophase band of microtubules (arrows) may be seen together with darkly staining vesicles and smooth endoplasmic reticulum. × 56,000.

Fig. 5. Cell treated similarly to that shown in Fig. 4. The cell is at c-metaphase. Microtubules extend for a short distance from the point of attachment to the chromosome. × 32,000.
Fig. 6. Cell treated similarly to that shown in Fig. 4. The edge of an uncompleted cell plate is shown: Very few microtubules are present. × 70 000.

Fig. 7. Cell treated with heavy water (99%) for 5 h. The cell is at preprophase. The preprophase band of microtubules shows an unusually large number of microtubules. These extend up to 15 rows deep into the cytoplasm. The orientation of the microtubules is much more variable than in untreated cells. × 48 000.
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Fig. 8. Higher magnification of a cell treated similarly to that shown in Fig. 7. The disorientation of the microtubules in the preprophase band is very apparent. Some smooth profiles of endoplasmic reticulum can be seen among the microtubules of the band. × 62000.

Fig. 9. Cell treated similarly to that shown in Fig. 7. During mitosis the area of the nucleoplasm between the chromosomes contains a very large number of microtubules. × 40000.
Fig. 10. Cell treated similarly to that shown in Fig. 7. At the edge of the cell plate a large number of microtubules are visible. × 72,000.

Fig. 11. Cell treated with a solution of heavy water (70%) containing colchicine (0.1% w/v) for 3 h during the 11-h recovery period after synchronization with 5-aminouracil. The cell is at prophase. The prophase band of microtubules contains few microtubules but these extend well into the cytoplasm (arrows). Compare with Figs. 4 and 7. × 50,000.
Polymerization of microtubules