MICROTUBULE-ORGANIZING CENTRES DURING
THE CELL CYCLE OF 3T3 CELLS

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SUMMARY
When Swiss 3T3 cells are treated with colcemid for 60 min (0.5–2 µg/ml), although the cytoplasmic network of microtubules is considerably reduced, a significant but variable number of microtubules remain. These residual microtubules appear to converge on a single, usually ciliated, organizing centre. In contrast, on removal of the colcemid, microtubule regrowth takes place from two organizing centres in approximately 50% of the cells. Surprisingly, whether a cell has one or two organizing centres during recovery does not depend on its position in the cell cycle, though recovery takes place earlier in serum-stimulated cells than in serum-starved quiescent ones. It seems likely that prior to colcemid treatment, cells contain two potential organizing centres only one of which is normally active, the inactive one becoming active after exposure to the drug.

INTRODUCTION
The two-transition model of the cell cycle, put forward as a purely formal explanation of certain puzzling aspects of mammalian cell-cycle kinetics (Brooks, Bennett & Smith, 1980; Brooks, 1981), shows many parallels with the biogenesis of mitotic centres in cleaving sea-urchin eggs (Mazia, Harris & Bibring, 1960). Mitotic centres – the entities responsible for forming the spindle poles – arise, in metazoans, from a region of the cytoplasm containing the centrioles (Fuge, 1977) and in interphase this region appears to organize the extensive network of cytoplasmic microtubules (Osborn & Weber, 1976; Brinkley, Fuller & Highfield, 1976; Frankel, 1976; Albrecht-Buehler & Bushnell, 1980; Brinkley et al. 1981). We have been interested therefore in whether the number of such microtubule-organizing centres (MTOCs) changes during the interphase portion of the cell cycle. That this might be so was suggested by the observations of Watt & Harris (1980) that growing cells had either one or two MTOCs, over 40% having two – a fraction far higher than the proportion of cells engaged in mitosis itself.

To determine the number of organizing centres as a function of cell cycle progress, we have used the now standard technique of disrupting microtubules with colcemid, removing the colcemid and fixing the cells just as the microtubules begin to reform (Brinkley et al. 1976; Osborn & Weber, 1976; Frankel, 1976). The MTOCs are then visualized using immunofluorescence microscopy after ‘staining’ with antibody to tubulin. They appear as bright spots at the focus of numerous short, radiating microtubules. Surprisingly, we have found that roughly 50% of quiescent 3T3 cells have two MTOCs (practically all of the remainder having one) and this frequency...
does not change when the cells are stimulated to resume proliferation. Thus in 3T3 cells, the number of functional organizing centres does not double as the cells enter S phase. These results differ significantly from those of Brinkley and colleagues (Brinkley, Cox & Fistel, 1980a; Brinkley et al. 1980b, 1981), who reported that the number of MTOCs per cell increased from one to two as the cells progressed from G₁ to S phase. The discrepancy may have arisen because many microtubules can remain in the presence of colcemid, especially in quiescent cells, and these residual microtubules converge to a single point within the cytoplasm, which is easily mistaken for a centre of microtubule regrowth. Since recovery from colcemid begins earlier in stimulated than in quiescent cells, the number of apparent organizing centres in the two cases can differ depending on the exact time of fixation.

MATERIALS AND METHODS

Cells

Swiss 3T3 cells (Todaro & Green, 1963) from two sources were used with similar results. One clone, 3T3/4A-C5, has been described before (Brooks, 1977); the other was obtained from Flow laboratories, Irvine, Scotland, recloned before use and given the designation 3T3/D5. Both clones were selected for their low saturation density and typical 'cobblestone' appearance in confluent cultures, though the D5 clone was slightly less serum-dependent than the 4A-C5 clone. Stocks of cells at low passage were maintained in liquid nitrogen and new ampoules recovered at approximately 8-week intervals. The cells were subcultured twice weekly using 0.05% trypsin (Difco) in 0.015% EDTA and propagated in Dulbecco's modification of Eagle's medium (Morton, 1970) supplemented with 10% new-born calf serum (Gibco, Paisley, Scotland).

To prepare quiescent cultures, 3 x 10⁴ 3T3/D5 or 5 x 10⁴ 3T3/4A-C5 cells were seeded into the 16 mm diameter wells of Linbro multidish trays (Linbro 24-16-TC) in 1 ml of medium containing 0.25 or 0.3-0.5% new-born calf serum, respectively. Each well contained a 13 mm diameter glass coverslip. The cultures were used 3 days after plating, when more than 98% of the cells are in G₁ (e.g., see Brooks, 1975, 1977). When 'stimulated' cells were required the coverslips were transferred to 3 cm dishes containing 2 ml medium supplemented with 10% dialysed serum and 25 μM-inosine (Brooks, 1975, 1977). Under these conditions cells start to enter S phase about 14 h after serum addition and begin to reach mitosis after approximately 24 h.

Antibodies

The anti-tubulin antibody was generously provided by R. W. Tucker (John Hopkins Medical School, Baltimore). It was raised in rabbits against vincristine-induced tubulin paracrystals from unfertilized sea-urchin eggs (Sato, Ohnuki & Fujiwara, 1976). It was used at a dilution of 1:10 or 1:5.

For experiments not involving photographs, the second antibody was fluorescein isothiocyanate conjugated F(ab')₂ goat anti-rabbit immunoglobulin (Ig) kindly donated by M. Ritter & R. Sutherland (I.C.R.F.) and used at a dilution of 1:150. For photographic purposes, the second antibody was tetra-methyl rhodamine isothiocyanate conjugated F(ab')₂ sheep anti-rabbit F(ab')₂, generously given by M. Ritter and R. Morris and used at approximately 60 μg/ml.

Other reagents

Colcemid (demethylcolcine) was supplied by Sigma and dissolved in 0.01 M-HCl at 1 mg/ml. Stock solutions were further diluted with water to 0.1 mg/ml and stored frozen at −20°C. Colcemid was also obtained from Gibco as a solution containing 10 μg/ml in Hanks' balanced saline. This was stored at 4°C. No differences were noted between the two supplies. Colcemid solutions were kept in the dark to avoid photo-inactivation.

[^3]H]thymidine ([methyld-3H]) was obtained from New England Nuclear at a specific activity of 20 Ci/mmol. It was used at 1 or 2 μCi/ml without the addition of unlabelled thymidine.
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Triton X-100 was obtained from Sigma, and formaldehyde (38%) from Fisons, Loughborough, England.

Colcemid treatment

To reveal MTOCs, cells on coverslips were treated with colcemid (generally 0.5 µg/ml, 1-4 µM) for 60 min in the dark. Coverslips were then rinsed twice in warm (37°C) medium to remove colcemid, returned to colcemid-free medium and fixed for immunofluorescence 30 min later, or as required.

Indirect immunofluorescence

Cells were fixed according to the procedure described by Heggeness, Wang & Singer (1977). Briefly, coverslips were immersed in 3% formaldehyde in Dulbecco's phosphate-buffered saline (PBS; solution A, Dulbecco & Vogt, 1954) pre-warmed to 37°C, and then set at room temperature for 30 min. The coverslips were rinsed in PBS, treated with 0.1 M-glycine in PBS for 10 min to neutralize any remaining aldehyde groups and rinsed again in PBS. The cells were then permeabilized by placing the coverslips in 0.1% Triton X-100 in PBS for 2 min followed by thorough rinsing in PBS. Although this fixation procedure sometimes increased the non-specific nuclear fluorescence (obtained with the second antibody alone) it was preferred to other standard techniques, such as methanol at −20°C or permeabilization with acetone, because it gave superior preservation of microtubules, as noted by Heggeness et al. (1977).

For 'staining', coverslips were placed (cells uppermost) on damp filter paper inside a 9 cm Petri dish and 15 µl of anti-tubulin antibody was applied before the cells dried. The Petri dishes were then incubated in a humidified box at 37°C for 60 min, after which the coverslips were rinsed thoroughly by repeated dipping into PBS as described by Weber, Bibring & Osborn (1975). This procedure was then repeated after applying 15 µl of the fluorescein- or rhodamine-conjugated second antibody. Finally the coverslips were mounted onto slides in PBS/glycerol (50:50), and sealed with melted paraffin wax. Cells were viewed with a Zeiss R.A. microscope (63X planapochromatic phase objective, N.A. 1.4) equipped with appropriate filters for rhodamine or fluorescein fluorescence and using epi-illumination. Cells were photographed using Kodak Ektachrome film, ASA 400, and an exposure of 90 or 120 s.

Autoradiography

Cells not previously treated for immunofluorescence were first fixed with 'formal saline' (4% (w/v) formaldehyde, 0.5% (w/v) NaCl, 1.5% (w/v) Na₂SO₄ in H₂O) (Brooks, 1975). Cells were extracted with 5% trichloracetic acid and prepared for autoradiography exactly as previously described (Brooks, 1975) except that the emulsion was Ilford K5, the exposure 3-4 days and the developer Kodak D19 diluted 1:2 with water. Where coverslips had previously been used for immunofluorescence, they were first rinsed in water (×2), 70%, 95% and absolute ethanol, toluene (×2), dried and the procedure repeated, in order to remove residual PBS/glycerol, paraffin wax and immersion oil.

RESULTS

MTOCs and the cell cycle

In our initial experiments we verified that 60 min exposure to colcemid (0.5-1 µg/ml) followed by 30 min recovery allowed microtubule organizing centres (MTOCs) to be visualized as brightly fluorescent spider-like spots after staining with antibody to tubulin. In contrast to the report by Spiegelman, Lopata & Kirschner (1979) of multiple initiation sites in mouse fibroblasts, serum-stimulated 3T3 cells were observed to have either one or two organizing centres (Fig. 1a, b), less than 3% of the cells in general having more than two. Similar results have been obtained by others (Osborn & Weber, 1976; Frankel, 1976; Brinkley et al. 1976, 1980a,b, 1981;
Fig. 1. Microtubule organizing centres in 3T3 cells. A and B, quiescent cells stimulated with 10% serum for 21 h (see Materials and Methods); C, as for A and B, but no colcemid treatment; D, quiescent control; cells left in low serum concentration for a further 21 h. The colcemid treatment (A, B, D) was 0.5 μg/ml for 60 min followed by 30 min recovery. The [3H]thymidine-labelling indices for parallel coverslips were: 1.1%, quiescent; and 53%, serum-stimulated (21 h labelling period, duplicate determinations). Cells, 3T3/4A-CS. Bars, 10 μm.

Watt & Harris, 1980). Frequently, several intensely staining filaments (taken to be microtubules) were seen to radiate out from the organizing centres (Fig. 1A) but this was not invariably so (Fig. 1B). For comparison, the appearance of cells not treated with colcemid is shown in Fig. 1c. Although the intensity of staining was always greatest in a region adjacent to the nucleus (often containing a cilium), it was rarely possible to detect discrete organizing centres without first disrupting the pre-existing microtubule network and then allowing a short period of recovery.

In the experiment shown in Fig. 1 (A, B) 47% of the cells had two MTOCs. In obtaining this value, multinucleate or giant cells (roughly 1 or 2% of the population) were excluded from the count—a convention adopted throughout. Since the [3H]thymidine-labelling index in parallel cultures was 53% we were tempted to speculate that the cells with two MTOCs were the ones in S phase. However, we subsequently found that a large proportion of a quiescent population (between 33 and 60% in different experiments) also had two MTOCs even though only 1% of the cells were in S phase (Fig. 1d). Furthermore, the frequency did not change with time after serum stimulation, as is shown in Fig. 2 for two independent clonal lines of 3T3.
Fig. 2. MTOC frequency against time after serum stimulation. Quiescent cells were stimulated with 10% serum and the MTOC frequency was determined at various times thereafter. Colcemid treatment was 0.5 μg/ml (A) or 0.6 μg/ml (B) for 60 min with 30 min recovery. Each point represents a separate determination and is derived from a count of approximately 250 cells (immunofluorescence) or 500 cells (autoradiography). As will be seen later in Fig. 5, very few cells had no scoreable MTOC and these were ignored for the present purposes. A, 3T3/4A-CS; immunofluorescence and autoradiography performed on the same coverslips; B, 3T3/D5; immunofluorescence and autoradiography performed on parallel sets of coverslips. (O) 2 MTOC/cell; (△) >2 MTOCs/cell; (●) [3H]thymidine-labelling index. Label added with the serum except for the zero point, which received [3H]-thymidine for 90 min (i.e. beginning with the colcemid treatment) but no serum addition.
Evidently, whether a cell has one or two functional MTOCs (following colcemid removal) does not depend on its position in the cell cycle.

Colcemid-resistant microtubules

During the course of these experiments we were surprised to find a substantial number of microtubules remaining after 60 min exposure to 0.5 µg/ml of colcemid (Fig. 3A, B, D, E) – rather more, in fact, than generally seen 30 min after the colcemid had been removed (cf. Fig. 1). The same result was obtained if the period of exposure was increased to 90 min (not shown) or the colcemid concentration raised to 2 µg/ml (Fig. 3C, F). There was considerable heterogeneity between cells in the number of residual microtubules (compare Fig. 3A with B and D with E). Despite this, it was apparent that quiescent cells possessed rather more residual microtubules in general than stimulated cells – an impression confirmed by several independent observers. This is also evident when comparing Fig. 3A with D (which may be taken as representative of the 'average' appearance of quiescent and stimulated cells, respectively), though given the heterogeneity between cells (Fig. 3A–C, D–F) it is difficult to substantiate this impression with photographs of what are inevitably selected fields.

Organization of colcemid-resistant microtubules

As can be seen in Fig. 3, the residual microtubules frequently converged to a point within the cytoplasm. These foci often (especially in quiescent cells) bore a cilium (Osborn & Weber, 1976; Tucker, Pardee & Fujiwara, 1979; Albrecht-Buehler & Bushnell, 1980) but were not otherwise well-stained. Thus, in the absence of a cilium they appeared as little more than the intersection of several microtubules with no accumulation of stainable material at the intersection itself (Fig. 3D). As these foci of residual microtubules might conceivably reflect the activity of organizing centres functioning before exposure to colcemid, or perhaps even partially active in the presence of colcemid, we have attempted to determine the number of such foci per cell. The results of one such experiment are given in Table 1. We adopted the minimum convention that to qualify for inclusion a focus must contain a prominent cilium and at least three convergent microtubules or, in the absence of a cilium, at least five intersecting microtubules. The latter condition was necessary in order to avoid including cases of two microtubule fragments crossed by chance – a common hazard as many cells contained numerous short fragments. A few cells contained many residual microtubules with no clear-cut foci and these were placed in a category labelled 'Too Difficult'. Examples of such cells may be found in Fig. 3 (C, E). Other cells had no residual microtubules whatever or a small number of non-convergent fragments (e.g. Fig. 3E) and these were placed in the '0 Foci' class. In no case does the Too Difficult category represent a substantial proportion of the population, though the 0 Foci class is larger for serum-stimulated as compared to quiescent cells (Table 1). To some degree this is quantitative support for the subjective statement made earlier that stimulated cells generally have fewer and shorter residual...
microtubules than quiescent cells. Of the cells that could be scored as having foci, only a very small proportion had more than one and this was unaffected by either growth state or colcemid concentration (Table 1).

**Regrowth of microtubules after colcemid removal**

As seen in Table 1, in the presence of colcemid the residual microtubules converge
Table 1. Organization of residual microtubules in the presence of colcemid

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For quiescent cultures, cells (3T3/C5-4A) were seeded onto coverslips in 0.5% calf serum 4 days before fixation. Stimulated cells were prepared in the same way but were placed in medium containing 10% dialysed calf serum and 25 μM-inosine 21 h before fixation. Colcemid was added 60 min before fixation. Too Diff., category labelled 'Too Difficult'.

almost exclusively to a single focus, yet when the colcemid is removed regrowth in many cells takes place from two centres. The time-course of recovery is illustrated photographically in Fig. 4 and graphically in Fig. 5. The behaviour of quiescent and stimulated cells is similar except in one important respect: recovery begins significantly earlier in the stimulated cultures. In other experiments no change was observed up to 15 min after removal of the colcemid but by 20 min, in the experiment shown in Fig. 4, many stimulated cells possess two bright spots (Fig. 4E, Fig. 5). This is not seen in quiescent cultures until 25 min after colcemid removal (Fig. 4B, Fig. 5), and at each time point thereafter the organizing centres appear less well-developed than in the corresponding stimulated cells. As recovery proceeds, the impression has been gained repeatedly that the residual microtubules become fewer, shorter and more fragmented (especially in stimulated cells), but before this happens, in practically all cells with two MTOCs the residual microtubules are preferentially associated with only one centre (Fig. 4B, C, E, F). Nevertheless, as recovery continues, in cells with two MTOCs the diameter of the halo around each is similar at any given time suggesting equivalent rates of microtubule regrowth (Fig. 4D, G, H).

Fig. 4. Time-course of recovery from colcemid. Cells were treated with colcemid (0.5 μg/ml for 60 min) and fixed at various times after its removal: A and E, 20 min; B and F, 25 min; C and G, 30 min; D and H, 40 min. A-D, quiescent cells; E-H, stimulated cells (10% serum added 20 h before colcemid). Cells, 3T3/4A-C5. Bars, 10 μm.
Fig. 4
Fig. 5. Time-course of recovery from colcemid – quantitative data from the experiment shown in Fig. 4. Cells were classified as having 0, 1, 2 or >2 MTOCs per cell or as being ‘Too Difficult’. Each point represents a separate determination with duplicates joined by vertical bars. The lines drawn are through the mean values. For clarity, the >2 class has been omitted as the points lie amidst those of the 0 or Too Difficult classes. Approximately 150 cells were scored for each point. (Δ, O, □) quiescent cells (Q); (△, ●, ■) stimulated cells (10% serum for 21 h) (S). α (O, ●) 2 MTOCs; (△, △) 0 MTOCs. α (O, ●) 1 MTOC; (□, ■) ‘Too Difficult’ category.
DISCUSSION

MTOCs and the cell cycle

The initial purpose of this study was to determine whether the number of microtubule-organizing centres functioning after colcemid removal changed as the cells progressed towards cell division from quiescence. For the Swiss 3T3 cells used this is clearly not the case. In view of the association between centrioles and the cytoplasmic MTOCs of mammalian cells (Brinkley et al. 1980a, 1981), this failure to detect MTOC duplication is surprising since centrioles are known to duplicate in the middle of interphase (Robbins, Jentsch & Micali, 1968; Rattner & Phillips, 1973; Vorobjev & Chentsov, 1978, 1982; Kuriyama & Borisy, 1981). More specifically, for the cell type used here, centriole replication begins approximately 4 h after the start of S phase (Alvey, 1982), but this is evidently not associated with any obvious change in MTOC activity. Nevertheless, MTOCs – like other cellular structures – must duplicate during the cell cycle if their number is to be conserved. It must be presumed, therefore, that the early stages in the process of duplication (perhaps correlated with procentriole formation) are simply not resolvable by immunofluorescence microscopy. Indeed, since the experiment in Fig. 2 was continued to a time when the most advanced cells begin to reach mitosis (24 h), we now suspect that tangible signs of duplication, as far as immunofluorescence is concerned, do not occur until after mitosis. With this in mind, it seems likely that the 'duplication' of MTOCs reported by Brinkley et al. (1980a, b, 1981) for synchronized, rapidly cycling Chinese hamster ovary (CHO) cells actually represents the completion of a process initiated in the previous cell cycle rather than a response to the start of the current S phase as suggested by them. For example, after mitosis cells inherit a mature centriole together with its associated daughter centriole, and these separate (or disorientate) during G1 thereby completing the centriole cycle (Kuriyama & Borisy, 1981). Unfortunately, our own attempts to follow MTOC behaviour in mitotically selected CHO cells have been unsuccessful as our cells attach poorly to glass coverslips. As a result, the cells are not well-spread and their poor optical properties make detection of MTOCs difficult; particularly, soon after plating, and hence early in the cycle.

Although the number of apparent MTOCs is not related in any straightforward sense to cell-cycle progress, the fact that some cells have one and others have two deserves comment. The simplest explanation is that practically all cells actually have two potentially functional centres but only those that are sufficiently far apart can be scored as two. This is consistent with the fact that quiescent (as well as cycling) cells possess two adult centrioles (Tucker et al. 1979; Alvey, 1982). However, at 30 min after removal of colcemid, the MTOCs are roughly 2–3 μm in diameter and must obviously be more than 3 μm apart to be resolved as two. Despite this, Alvey (1982) has found that 90% of mononucleate 3T3 cells not treated with colcemid (stimulated or quiescent) have their two adult centrioles within 2 μm of each other – a conclusion that stems from an examination of the centrioles in more than 500 cells by serial-section electron microscopy. Evidently, colcemid must cause centriolar separation, or
not all mammalian (cytoplasmic) MTOCs contain centrioles. In this connection, it is interesting that after comparing the effects of cold treatment with those of microtubule poisons, Watt and colleagues (Watt & Harris, 1980; Watt, Sidebottom & Harris, 1980) concluded that potential MTOCs are normally aggregated within the cell but can be split apart by agents such as colcemid. Similar results have also recently been obtained by Sherline & Mascardo (1982).

**Microtubules remaining in the presence of colcemid**

It has been known for many years that some microtubules may remain in the presence of colcemid (Brinkley, Stubblefield & Hsu, 1967; Osborn & Weber, 1976). Nonetheless, the number remaining after 60 min exposure to up to 2 μg/ml of colcemid was surprising as there seemed to be rather more remaining in the presence of the drug than 30 min after its removal. We have not, as yet, attempted to obtain objective evidence of this apparent decrease, but if real it suggests some sort of reorganization of the MTOC during the process of recovery from the drug.

That stimulated cells should have fewer and shorter residual microtubules than quiescent cells is less surprising. It has been shown recently that ATP depletion as a result of, say, azide poisoning prevents the disruption of microtubules by colcemid, vinblastine or nocodazole (Bershadsky & Gelfand, 1981; DeBrabander *et al.* 1981) and quiescent cells have a smaller ATP pool than stimulated cells (Grummt, Paul & Grummt, 1977). A further possibility is that stimulated cells, being more motile than quiescent ones (Castor, 1971), might reorganize their cytoskeleton more frequently — the microtubule reassembly step during reorganization being colcemid-sensitive. Because locomotion has a pronounced stochastic component (Gail & Boone, 1970) — some cells remaining stationary for comparatively long periods (unpublished observations) — this might also account for the heterogeneity observed in the number of residual microtubules per cell.

**Microtubule organization in the presence of and during recovery from colcemid**

The residual microtubules in colcemid-treated cells are focussed on a single, generally ciliated, centre in both quiescent and serum-stimulated cultures. Nevertheless, when the colcemid is removed, recovery takes place from two foci in 50% of the cells. In many cases the two centres remain distinguishable insofar as the long residual microtubules are preferentially associated with only one of them (Fig. 4B, C, E, F). However, as recovery proceeds, the two centres each develop a halo of new microtubules of similar diameter and intensity suggesting that, whatever their original differences, they eventually acquire a comparable degree of activity.

As for timing, we have shown that recovery begins significantly earlier in stimulated cells than in quiescent ones. Although we have no explanation of this other than the generally higher level of metabolic activity (and perhaps higher tubulin concentration) in growing cells, it is clear that a time can be found when quiescent cells show only one apparent organizing centre while many stimulated cells contain two. This may explain why Brinkley *et al.* (1980a,b, 1981) found only one MTOC per cell in
non-growing 3T3 and CHO cultures, i.e. their quiescent cells may have been fixed just before recovery had begun.

**Colcemid-induced activation of MTOCs?**

Finally, in view of our observation that residual microtubules in colcemid-treated cells are associated with only one of two potential centres, it is interesting that structural differences have been noted between the two adult centrioles of a cell. Thus, only one of them ever bears a cilium and it is this centriole that alone possesses basal feet (pericentriolar satellites) – apparently the principal sites of microtubule nucleation during interphase (Vorobjev & Chentsov, 1978, 1982; Albrecht-Buehler & Bushnell, 1980; Rieder & Borisy, 1982). Thus, in untreated cells, it seems likely that only one of the two centrioles is associated with extensive microtubule organization. If so, then since microtubule regrowth during recovery from colcemid takes place from two centres in many cells, it would appear that colcemid treatment may cause not only centriole separation (as discussed) but also activation of previously inactive centrioles – perhaps as a consequence of separation. The organization of colcemid-resistant microtubules may thus, ironically, provide a better reflection of normal organization than the appearance of cells during recovery from the drug. At any rate, it can no longer be assumed that colcemid treatment is entirely neutral, as far as MTOC activity is concerned. It is perhaps interesting to speculate that activation of previously inactive centres might be the basis of the potentiation of certain growth factors by agents such as colcemid (Otto, 1982).

We are greatly indebted to Robert W. Tucker for his generous gift of the anti-tubulin antibody, and to Mary Ritter and Robert Sutherland for kindly supplying the fluorescent second-antibody preparations. We are also grateful to W. Bessant for his skill and patience in preparing the black and white prints from the original colour slides.

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(Received 19 July 1982)