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Effect of microtubule inhibitors on the expansion of hypoblast and margin of overgrowth of chick blastoderms

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SUMMARY

The effect of the microtubule inhibitors, Nocodazole and taxol, was studied on the expansion of fragments of chick hypoblast (8 to 10 h incubation) and of margin of overgrowth (24 h incubation) cultured on artificial substrata and on the epiboly in intact blastoderms (24 h incubation).

Immunocytochemical staining of these cells with antiserum against tubulin showed that 1 μg Nocodazole/ml caused disassembly of microtubules, and that 1 μg taxol/ml led to increased but unordered assembly. The solvent dimethylsulphoxide had no effect. At these concentrations both microtubule inhibitors led to rapid arrest of the expansion of fragments of hypoblast and of margin of overgrowth in culture, and of the epiboly in intact blastoderms. Time-lapse films showed that inhibition of expansion in both situations was reversible within 2 h after removal of the drugs. Phase-contrast microscopy showed remarkably little difference between the morphology of treated as compared to untreated cultures. Measurements of the height of the cells on sectioned fragments of margin of overgrowth showed no differences between treated and untreated cultures. These results suggest that the cytoplasmic microtubule complex is important both for epiboly and for the migration of hypoblast cells in the chick blastoderm. The mechanisms of this microtubule-related migration are not understood.

INTRODUCTION

The role of cytoplasmic microtubules (MT) in the directional migration of cells is not fully understood. It is widely accepted that an intact cytoplasmic microtubule complex (CMTC) (Brinkley, Fuller & Highfield, 1975) is necessary for the directional migration of solitary fibroblasts (Vasiliev & Gelfand, 1976) as it confines the ruffling activity to one pole of these cells.

Most authors believe that the integrity of the CMTC is essential for the orientation of migration in macrophages and some classes of leucocytes but not for migration per se in these cells (Wilkinson, 1976; Bäck, Bandmann, Norberg

& Söderström, 1978). Controversy, however, exists about the role of the CMTC in epitheloid cells which move as a sheet. According to Vasiliev and coworkers (Vasiliev & Gelfand, 1976; Vasiliev *et al.* 1975; Domnina, Pletyushkina, Vasiliev & Gelfand, 1977) the ruffling activity at the leading edge of cells in sheets is not dependent on microtubules and, therefore, directional migration can continue in the presence of microtubule inhibitors (MTI). Other authors (Downie, 1975; De Brabander *et al.* 1976; Selden, Rabinovitch & Schwartz, 1981; Middleton, 1982) have shown that MTI interfere with the capacity of epitheloid cells to perform directional migration. Among the latter authors, however, different opinions exist about the mechanisms of MT-mediated cell migration (Review by Mareel & De Mets, 1984).

In the present experiments we have used hypoblast and margin of overgrowth (i.e. the most peripheral cells of the area opaca) from chick blastoderms to study the effect of MTI on directional migration. These cells are known to migrate like epitheloid sheets both in the blastoderm and *in vitro* (New, 1959; Bellairs, Boyde & Heaysman, 1969; Downie & Pegrum, 1971; Sanders, Bellairs & Portch, 1978; Ireland & Stern, 1982). Indeed, the expansion of the blastoderm (epiboly) is largely dependent on the migratory activity of the cells of the margin of overgrowth. Two types of MTI with different mechanisms of action were used: the synthetic drug Nocodazole (De Brabander *et al.* 1976) which interferes with the assembly of MT through binding to tubulin dimers (Hoebeke, Van Nijen & De Brabander, 1976); the alkaloid taxol which causes an increased but unordered assembly of MT through lowering of the critical concentration of tubulin (De Brabander *et al.* 1981). To ensure that Nocodazole and taxol at the concentrations used interfered with MT assembly/disassembly in these particular cells we have carried out immunocytochemical staining with an antiserum against tubulin.

MATERIALS AND METHODS

Blastoderms

Hens' eggs from commercial stock were incubated for either 8–10 h or for 24 h to reach stage XIV of Eyal-Giladi & Kockav (1976) or stage 4–6 of Hamburger & Hamilton (1951) respectively. Blastoderms were explanted ventral side up in accordance with New's technique (1955) and yolk was removed with balanced salt solution.

Observations on fragments

For observations on fragments, hypoblast (from stage XIV of Eyal-Giladi & Kockav, 1976) and margin of overgrowth (from stage 4–6 of Hamburger & Hamilton, 1951) were dissected from the blastoderm with stainless steel needles in balanced salt solution using a stereomicroscope (×25). The margin of overgrowth was identified as the translucent peripheral rim of the area opaca which

was attached to the vitelline membrane. Fragments of hypoblast (four to six fragments from each blastoderm) or from the margin of overgrowth (approximately the same size as hypoblast fragments) were explanted in the following culture vessels: on a square coverslip in a 60×15 mm plastic Petri dish (Falcon, Oxnard, Ca) for immunocytochemical staining and for measurements of the height of cells, in 25 cm^2 (30 ml) plastic culture vessels (Falcon, Oxnard, Ca) for time-lapse cinemicrophotography; in Nunclon Delta SI 24 wells multidishes (Nunc, Roskilde, Denmark) for measurement of diameters. In all experiments Minimum Essential Medium Eagle (modified) with non-essential amino-acids (MEM, Rega I, Gibco Europe, Paisley, Scotland) plus 10 % foetal bovine serum and 0·05 % L-glutamine was used (hereafter called culture medium). All fragments were preincubated at 37 ° for 3 to 4 h with a drop of culture medium to allow their attachment to the substrate. After addition of 500 μl (multidishes), 2 ml (Petri dishes), or 5 ml (25 cm^2 flasks) culture medium they were kept for a further 10–11 h, bringing the total period of preincubation to 14 h. Then, cultures were incubated with Nocodazole, with taxol, with the solvent of Nocodazole and taxol, with ethylene diamine tetra acetate (EDTA) or with culture medium. For open culture vessels a CO_2-incubator (5 % CO_2 in air, 100 % relative humidity) was used. EDTA was used at 0·03 % (w/v) in Ca^{++}- and Mg^{++}-free balanced salt solution. Nocodazole (gift from M. De Brabander, Janssen Pharmaceutica, Beerse, Belgium) and taxol (gift from the Drug Synthesis and Chemistry Branch, National Institutes of Health, Bethesda, Md) were dissolved in dimethylsulphoxide (1 mg/ml) and further diluted in culture medium to a final concentration of 1 μg/ml. This concentration was chosen on the basis of previous experiments (Storme & Mareel, 1980; Mareel *et al.* 1982). Control cultures were incubated with or without addition of dimethylsulphoxide (0·1 % v/v). To study reversibility treated cultures were washed three times in an excess of culture medium prewarmed to 37 °. For immunocytochemistry coverslips were fixed in glutaraldehyde (10 min, 1 % in cacodylate buffer, 0·1 M, pH = 7·4) and stained with an antiserum against tubulin (gift from J. De Mey and M. De Brabander, Janssen Pharmaceutica, Beerse, Belgium) following the unlabelled antibody enzyme method (De Mey *et al.* 1976).

For measurements of the mean diameters of explanted sheets we used a phase-contrast Diavert microscope (obj. ×4; Leitz Wezlar, G.F.R.) equipped with an eyepiece graticule (×10).

For time-lapse cinephotomicrography under a phase-contrast microscope (objectives ×6 and ×20) mounted in an incubator we used a Bolex-Paillard 16 mm camera equipped with an electronic time-lapse regulator (Wild, Heerbrugg, Switzerland). One frame was taken every 20 seconds and films were analysed at 18 frames per second.

For measurements of cell heights cultures were fixed and embedded for light microscopy following previously described methods (Van Peteghem, Mareel & De Bruyne, 1980). Radial 2 μm-thick sections were made through the periphery

of explants of margin of overgrowth and stained with haematoxylin-eosin. Between each 2 μm-thick sections 40 μm-thick slices were discarded to avoid measuring the same cells twice. Heights of cells were measured at the level of the maximal vertical diameter under a microscope (obj. ×100) using an eyepiece graticule (×10).

Observations on whole blastoderms

For observations on whole blastoderms each embryo was removed from the egg at about stage 4–6 of Hamburger & Hamilton (1951) and explanted *in vitro* using the technique of New (1955) in which the embryo is explanted on egg albumen. Each embryo was preincubated for 12 h before the first measurement was taken. The maximum diameter across the entire blastoderm was recorded using an eyepiece graticule in a Nikon dissecting binocular microscope at a magnification of ×10. Intact embryos were explanted in this way and measurements were made at intervals of 2 h up to a period of 12 h.

Nocodazole dissolved as described above was diluted with Pannet & Compton's (1924) saline to a final concentration of 1 μg/ml and added to half of these explants in sufficient quantity to cover the embryo. Dimethylsulphoxide solution (0·1 % v/v) was added to the remaining embryos which therefore served as controls. After 6 h treatment, all explants were washed three times in Pannet & Compton's (1924) saline, and the egg albumen in the culture dishes was replaced with fresh albumen.

In a subsidiary experiment the central region (i.e. the entire area pellucida plus some area opaca) was removed from each blastoderm (hereafter called operated blastoderms) before treatment.

Reproducibility

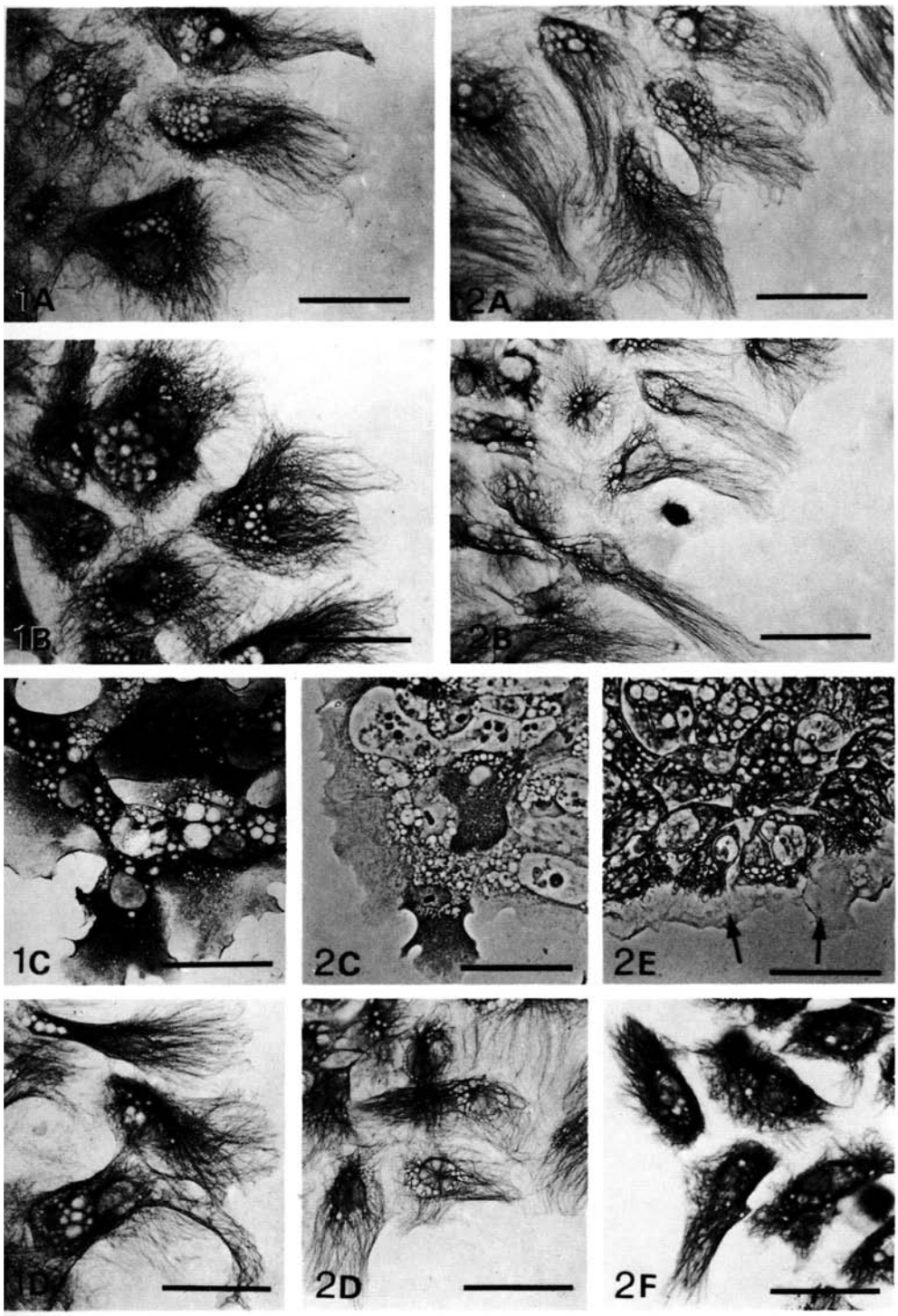
Numbers of cultures (n) and duration of treatment for each series of observations are given in the Results section. Each series of observations was repeated at least once.

RESULTS

Observation on fragments

Immunocytochemical staining with antitubulin of cultures, both of hypoblast and margin of overgrowth, fixed after 2 h (n = 7) or 4 h (n = 2) incubation in

Figs 1–2. Light micrographs of cultures of hypoblast (1) and margin of overgrowth (2), stained with an antiserum against tubulin. Fixation after 2 h incubation in culture medium (A), after 2 h in dimethylsulphoxide (B), after 2 h in Nocodazole (C), after 2 h in Nocodazole followed by washing and 2 h further incubation in culture medium (D), after 2 h in taxol (E), and after 2 h in taxol followed by washing and 2 h further incubation in culture medium (F). Scale bars = 50 μm. Arrows indicate unstained lamellae in taxol-treated cells.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culture medium showed long tracks of MT radiating from the perinuclear area and oriented towards the cell periphery in most interphase cells (Figs 1 and 2). Marginally positioned cells were radially oriented and tracks of MT extended into the peripheral lamellae. Our material did not, however, enable us to localize the MT organizing centre in all cells. Spindle MT were frequent in sheets of margin of overgrowth and occurred less frequently in hypoblast sheets. Incubation for 2 h (n = 9) with dimethylsulphoxide did not alter the immunocytochemical aspect of the MT complexes (Figs 1B and 2B). Incubation for 2 h with 1 μg Nocodazole/ml (n = 13) resulted in a diffuse staining of the cytoplasm. In hypoblast cells MT were totally absent (Fig. 1C); in cells from the margin of overgrowth a few short tracks of MT radiating presumably from the MT organizing centre were observed (Fig. 2C). Two hours after removal of Nocodazole (n = 9) MT were as in controls (Figs 1D and 2D).

After 2 h treatment with taxol (n = 3) all margin of overgrowth cells showed thick bundles of microtubules that were irregularly distributed. In contrast with controls, peripheral lamellae showed neither microtubules nor diffuse staining (Fig. 2E). Two hours after removal of taxol (n = 3) the CMTC was normal in some cells. Most cells had only partly recovered showing thick irregular bundles next to normal tracks of MT; unstained lamellae remained present in some cells (Fig. 2F).

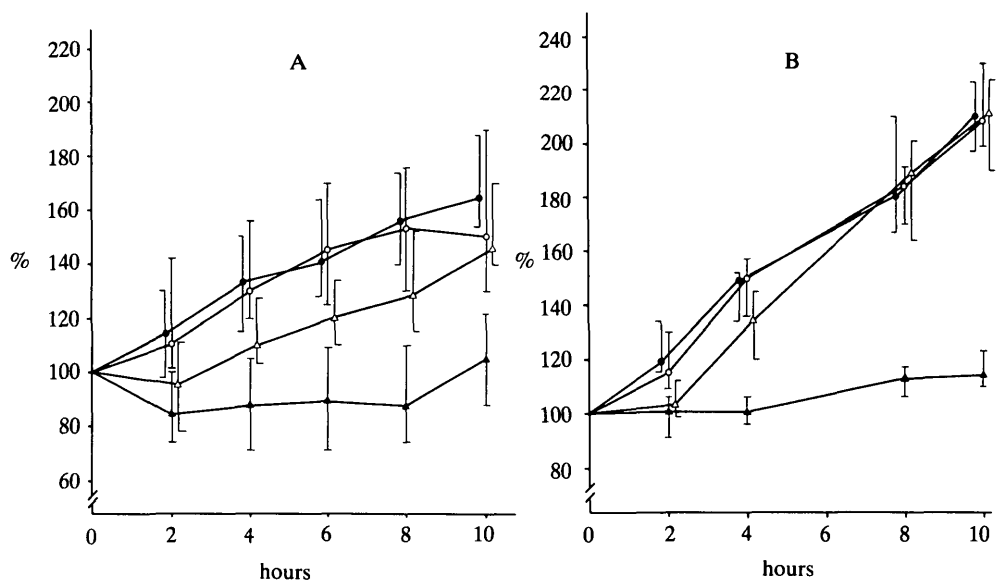


Fig. 3. Expansion of sheets of hypoblast (A) and margin of overgrowth (B), incubated with culture medium (○--○), with dimethylsulphoxide (●--●), with Nocodazole (▲--▲), and with Nocodazole for 2 h followed by washing and further incubation with culture medium (△--△). Ordinate: area covered by sheets as a % of area covered after preincubation (time zero); median and extreme values from seven to nine cultures. Abscissa: time of incubation.

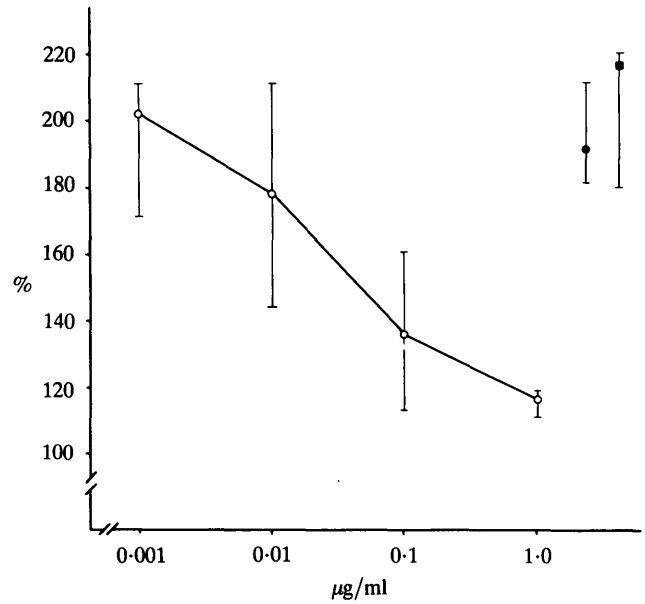


Fig. 4. Dose-response of inhibition of expansion of margin of overgrowth by Nocodazole (○). Controls were incubated with culture medium (■) and with the concentration of dimethylsulphoxide present at the highest concentration of Nocodazole (●). Ordinate: area covered by the cells after 4 h treatment as % of the area covered at the moment the drug was added; median and extreme values from five cultures. Abscissa: concentration of Nocodazole ($\log_{10}$).

The effect of Nocodazole on the expansion of sheets of hypoblast and margin of overgrowth is shown in Figs 3 and 4. It is apparent that Nocodazole arrests the expansion of both hypoblast and margin of overgrowth with or without an initial retraction of the sheet. The inhibition was reversible after at least 2 h of incubation with the drug. Dimethylsulphoxide on its own had no effect. With fragments of margin of overgrowth inhibition of expansion by Nocodazole was dose dependent (Fig. 4). Taxol affected the expansion of the margin of overgrowth in a similar way to Nocodazole. However, this inhibition was not reversible in all cultures as was obvious from the large variations between individual cultures (Fig. 5). Time-lapse films made at lower (objective ×6) magnification (n = 4) confirmed the inhibition of expansion by Nocodazole in individual cultures (Fig. 6). These observations demonstrated that with the margin of overgrowth the inhibition was reversible at least after 5 h treatment. In time-lapse films (Figs 7 and 8) made at higher (objective ×20) magnification (n = 21) cells at the edges of sheets of both hypoblast and margin of overgrowth appeared to migrate through extension of a lamella followed by leaping of the perikaryon. Ruffling was present at the leading edge of the lamella irrespective of whether or not cells were in lateral contact with their neighbours. Ruffling was not continuous and usually limited to cells at the edge of the sheet. Occasionally, ruffling was

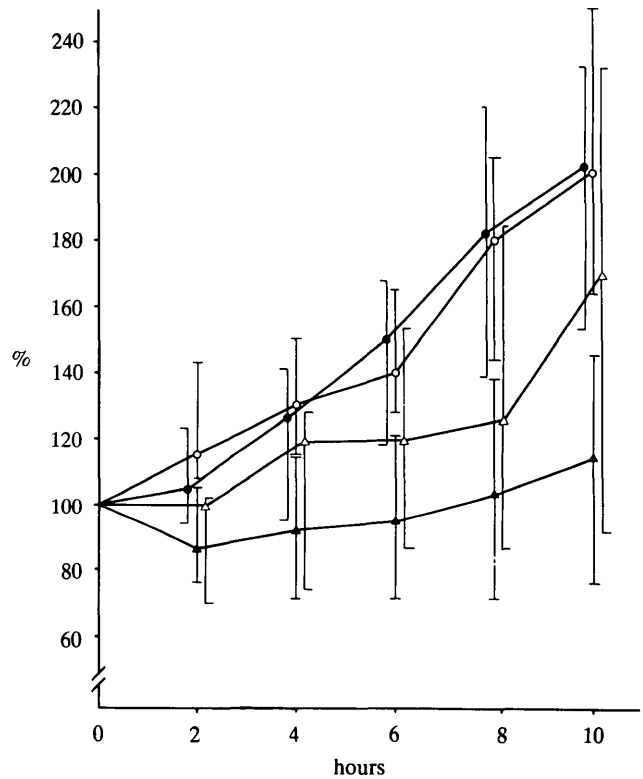


Fig. 5. Expansion of sheets of margin of overgrowth incubated with 1 μg taxol/ml (▲−−▲), with dimethylsulphoxide (●−−●), with culture medium (○−−○), and with taxol for 2 h followed by washing and further incubation with culture medium (△−−△). Ordinate: area covered by sheets as % of area covered after preincubation (time zero); median and extreme values from 7 cultures. Abscissa: time of incubation.

observed in second row cells, but it is known that such cells may sometimes shift to the edge of the sheet in chick cultures (Voon, 1980) and in some teleost (Kageyama, 1982) embryos. The bulk of yolk granules was found mostly at the trailing end of the nucleus. Apart from arrest of migration and, in some cases, retraction, a few changes were seen by phase-contrast microscopy in cells after addition of Nocodazole: these were redistribution of yolk around the nucleus, and shortening of the leading lamella. These alterations were not universally seen in all cultures and it was not possible to distinguish between control cultures and cultures treated with Nocodazole for 2 to 6 h solely by the inspection of phase-contrast micrographs (×200).

Treatment with Nocodazole of sheets of margin of overgrowth cultured on plastic did not alter the vertical diameter of the cells. Heights (mean ± S.D.) of cells (n = number of cells measured) were as follows: control cultures treated with dimethylsulphoxide (n = 28): 2·09 ± 0·45 μm; after Nocodazole for 2 h (n = 25): 1·76 ± 0·28 μm; after Nocodazole for 2 h followed by washing and

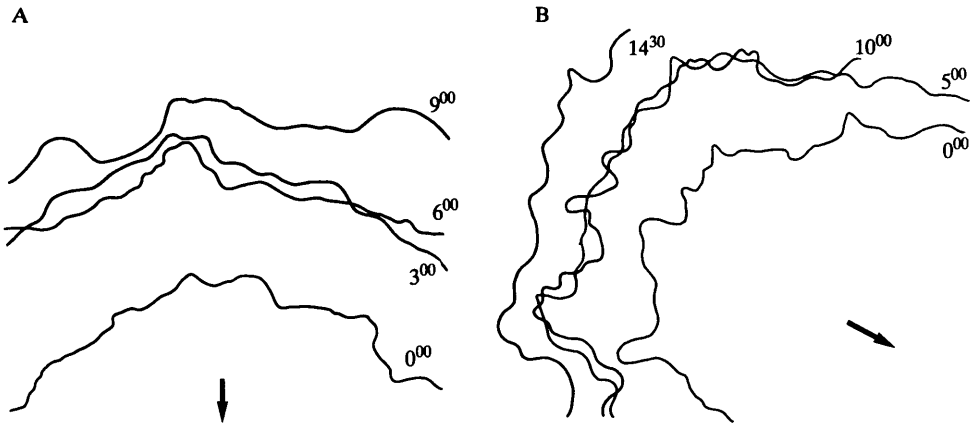


Fig. 6. Drawings of the periphery of expanding sheets from time-lapse films of cultures of hypoblast (A) and margin of overgrowth (B). Start of films after 14 h preincubation (0^{00}); addition of Nocodazole after 3 h to hypoblast (3^{00}) and after 5 h to margin of overgrowth (5^{00}), removal of Nocodazole after 3 h (6^{00}) and after 5 h (10^{00}) respectively, followed by further incubation in culture medium for $4\frac{1}{2}$ h (14^{30}).

further incubation in culture medium for 2 h (n = 41): $2{\cdot}53 \pm 1{\cdot}61\,\mu$m; after EDTA for 10 min (n = 29): $6{\cdot}38 \pm 1{\cdot}32\,\mu$m. Only cells treated with EDTA were significantly higher ($P < 0{\cdot}01$) than controls.

Observations on blastoderms

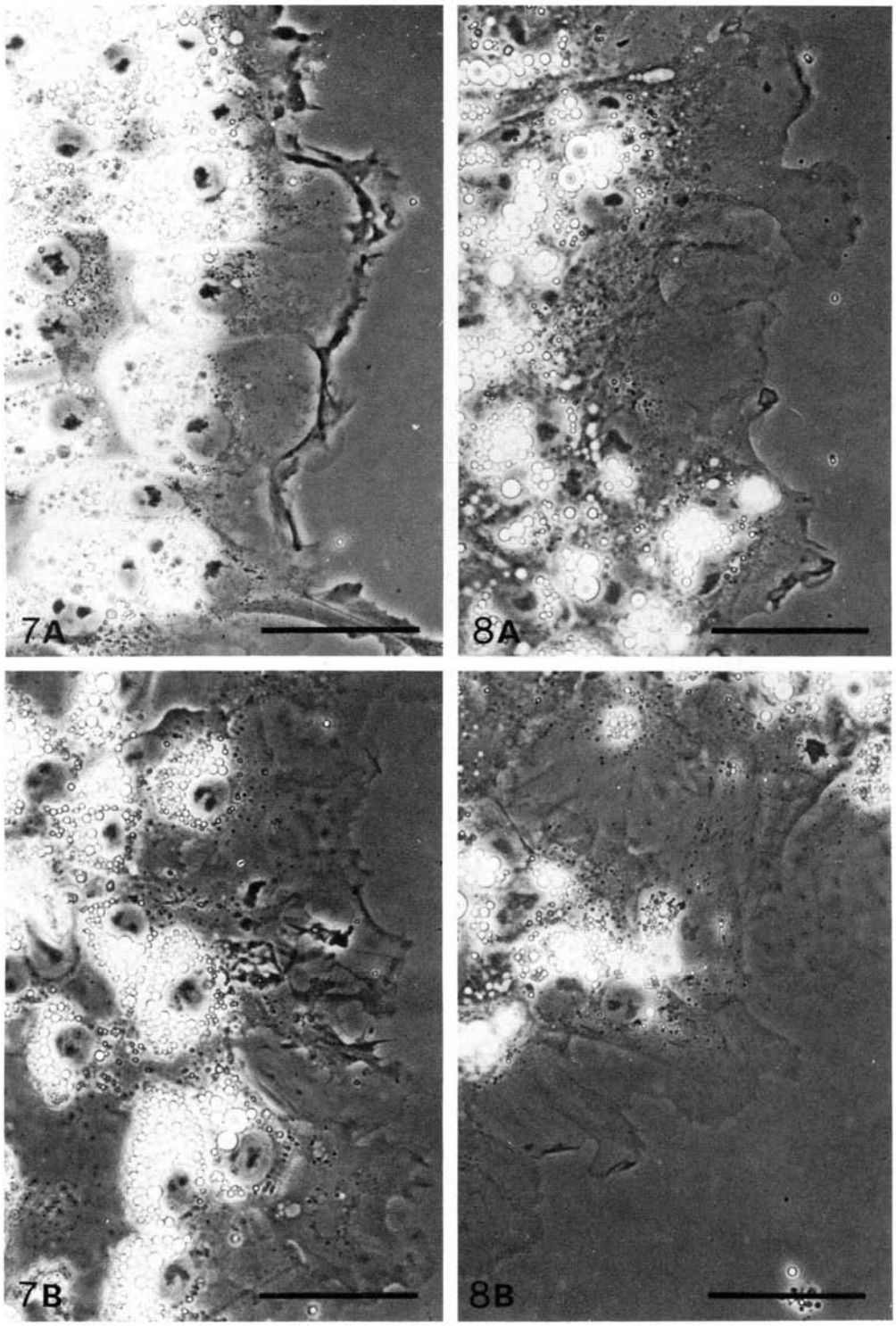
The results obtained from subjecting five intact blastoderms to Nocodazole are shown in Fig. 9A. Expansion usually was delayed when the blastoderm was treated with Nocodazole, and some retraction took place in two specimens. Once the Nocodazole had been washed away however, recovery took place in the space of 2 h. By contrast, the five controls treated with dimethylsulphoxide had expanded at a higher rate during the same period and appeared to be unaffected by the washing procedure.

Similar results were obtained with the operated blastoderms (Fig. 9B). With the Nocodazole treatment there was little expansion and sometimes retraction, but after washing at 6 h, the rate of expansion was increased in most specimens.

DISCUSSION

The present experiments show that the MTI we have used interfere with the expansion of sheets of hypoblast and margin of overgrowth on an artificial substrate and with the expansion of the margin of overgrowth in blastoderms on vitelline membrane. The latter observation is in agreement with data from Downie (1975) who has reported that colchicine inhibits the expansion of chick blastoderms in culture.

It is unlikely that inhibition of expansion of hypoblast and margin of overgrowth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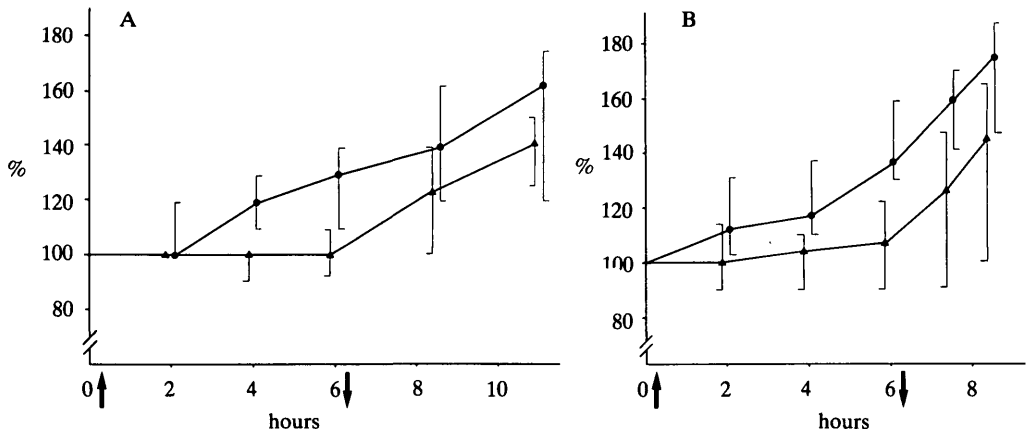

Fig. 9. Expansion of intact (A) and operated (B) blastoderms incubated with 1 μg Nocodazole/ml (▲--▲) or with dimethylsulphoxide (●--●). Ordinate: area covered by blastoderms as % of area covered after preincubation (time zero): median and extreme values from 5 cultures. Abscissa: time of incubation; arrows indicate addition (↑) and removal (↓) of drug.

in our experiments was due to cytotoxicity since it was rapidly and fully reversible. Neither could inhibition of expansion be ascribed to arrest of mitosis through interference of Nocodazole or taxol with the formation of the mitotic spindle for two reasons. First, our time-lapse films showed that arrest of expansion occurred within 10 to 15 min after addition of the drugs, a period during which only a minority of cycling cells could have reached metaphase. Second, inhibition of expansion was virtually the same with hypoblast as with margin of overgrowth although mitotic figures were uncommon in hypoblast cultures. Since the effects were observed using two MTI with different structures, different origin, and different mechanisms of action it can hardly be accepted that inhibition of expansion in both hypoblast and margin of overgrowth is related to a common side effect different from disturbance of assembly/disassembly of the CMTC. This opinion is supported by the results of immunocytochemical staining with an antiserum against tubulin.

The migration of the marginal cells seems to play a major role in the expansion of sheets of hypoblast and margin of overgrowth on artificial substrates, and of margin of overgrowth on vitelline membrane. The arguments are that these marginal cells preferentially attach to the substrate (New, 1959; Bellairs & New, 1962; Bellairs *et al.* 1969; Downie, 1975, 1976; Chernoff & Overton, 1977; Bellairs, Ireland, Sanders & Stern, 1981; Ireland & Stern, 1982) and that they

Figs 7–8. Stills from time-lapse films of sheets of hypoblast (7) and margin of overgrowth (8) before (A) and after 2 h of treatment (B) with Nocodazole. Scale bars = 50 μm.

produce a conspicuous leading lamella. This should not be taken to imply, however, that other cells in the sheet do not also participate in the expansion. Indeed, there is evidence of a minor contribution by the migratory activity of certain submarginal cells (Ireland & Stern, 1982).

Downie (1976) has argued that in the blastoderm tension in the inner part counteracts the expansion which is brought about by radial migration of the cells in the margin of overgrowth. The evidence is that the edge of the blastoderm retracts if it is released from the vitelline membrane. Downie (1975), who treated blastoderms with colchicine, concluded that the colchicine interfered with the expansion of the blastoderm because the cytoplasmic microtubules broke down and so became unable to maintain the flat shape of the inner cells, and consequently these cells were no longer able to resist the outwards pull of the edge cells. Tension therefore increased within the blastoderm so that further radial migration was arrested. Three points argue against this opinion.

First, as we have already seen, the evidence that tension exists in the inner part of the blastoderm comes exclusively from the fact that the edge cells retract if released from the vitelline membrane (Downie, 1976). It would seem therefore that it is the peripheral attachment of the blastoderm which promotes tension. Certainly, if there is no attachment at all, the blastoderm is flaccid and apparently lacks all tension.

Secondly, in a review De Brabander *et al.* (1977) have refuted the mechanistic concept that microtubules produce a rigid skeletal framework supporting cell shape in a direct way. In their opinion, the cell shape is primarily determined by the migratory behaviour of the cell, which is in turn influenced by external factors such as interaction with the substrate or with other cells.

Thirdly, in the present experiments, in contrast to Downie's (1975) observations, MTI affected the expansion of the margin of overgrowth on the vitelline membrane even when the inner part of the blastoderm was removed. A possible explanation for the discrepancy between our results and Downie's (1975) might be that different amounts of extraembryonic ectoderm or of yolky endoderm were left at the inner margin after dissection. Other explanations might be based on the fact that we used different MTI from Downie.

Whatever the mechanisms of microtubule regulated migration are, the present results as well as these published by Downie (1975, 1976) stress the importance of the CMTC both for epiboly and for the migration of hypoblast cells in the chick blastoderm.

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