

Ultrastructural features of pollen tubes of *Endymion non-scriptus* modified by cytochalasin D

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Summary. The cytochalasins, known as inhibitors of various processes involving motility in plant and animal cells, induce far-reaching structural changes in the cytoplasm and walls of pollen tubes without destroying the capacity for subsequent growth in normal media. The fine structure of tubes of *Endymion non-scriptus* modified by cytochalasin D suggests that the changes all stem directly or indirectly from the interruption of the long-range cyclosis along the tube axis, which is sustained throughout the period of normal growth. The elimination of this movement breaks down the pattern of flow responsible for the sorting-out process that maintains the characteristic zonation of organelles and other inclusions at the apex of the extending tube, and leads gradually to re-distribution of the vacuoles and membranes in the vegetative cell, the disposition of which is normally correlated with the longitudinally oriented flow pathways. Random local migrations of organelles and other inclusions of greater amplitude than is to be expected from Brownian movement continue in the tubes in the presence of cytochalasin D, indicating that the motility system is not wholly destroyed. Following the interruption of concerted axial movement, the polysaccharide wall-precursor bodies (P-particles), normally inserted into the wall mainly in the apical part of the tube during tip growth, gradually become dispersed throughout the tube and are incorporated in the wall at random, entering even into the intine of the parent pollen grain.

Key words: Pollen-tube structure – Intracellular movement – Tip-growth mechanism – Cytochalasin effects

Introduction

Franke et al. (1972) and Mascarenhas and La Fountain (1972) established that the cytochalasins, known as inhibitors of various processes involving motility in animal cells (Wessels et al. 1971), block the movement of organ-

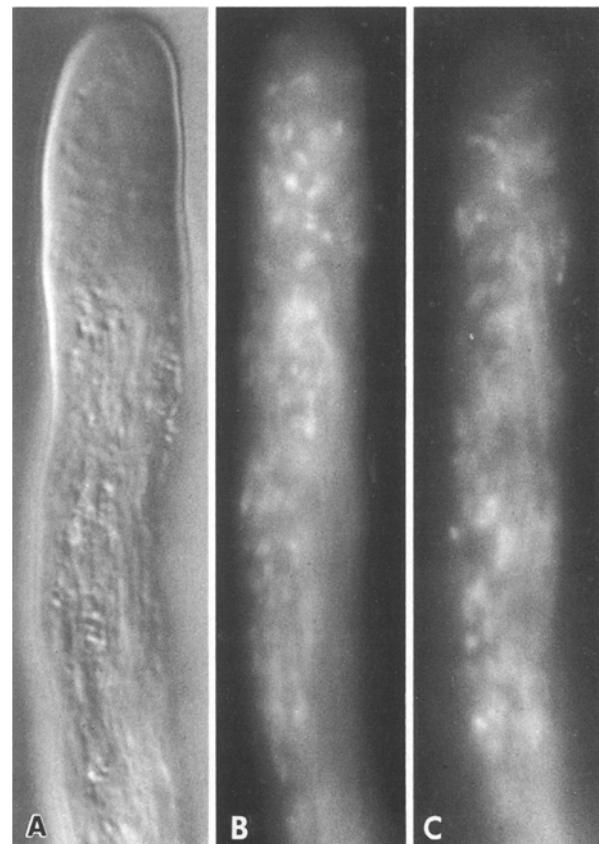


Fig. 1A–C. Streak micrographs showing the principal pathways of movement in a 45-min pollen tube of *E. non-scriptus* as observed in one focal plane. Approx. $\times 1500$. **A** Differential interference microscopy (DIC), 6-s exposure; **B** fluorescence micrograph, 3 s exposure, DIOC staining. Mitochondria approaching the tube tip are retarded and move in an indeterminate manner before entering a basipetal flow pathway; they can therefore be resolved as individual particulate bodies with so short an exposure period as this. Membranes of the endoplasmic reticulum, abundant in the sub-apical zone of the tube (compare Fig. 4) are not stained at the concentration of DIOC used. **C** Same field as in **B**, 8 s exposure. This longer exposure reveals the flow pathways more clearly, and the rapidity with which the mitochondria leave the apex once they have encountered a basipetally polarised pathway is well shown by the extended streaks at the centre right. Measurements from continuous recordings show that organelles in the basipetal traffic streams move at rates up to $5 \mu\text{m s}^{-1}$

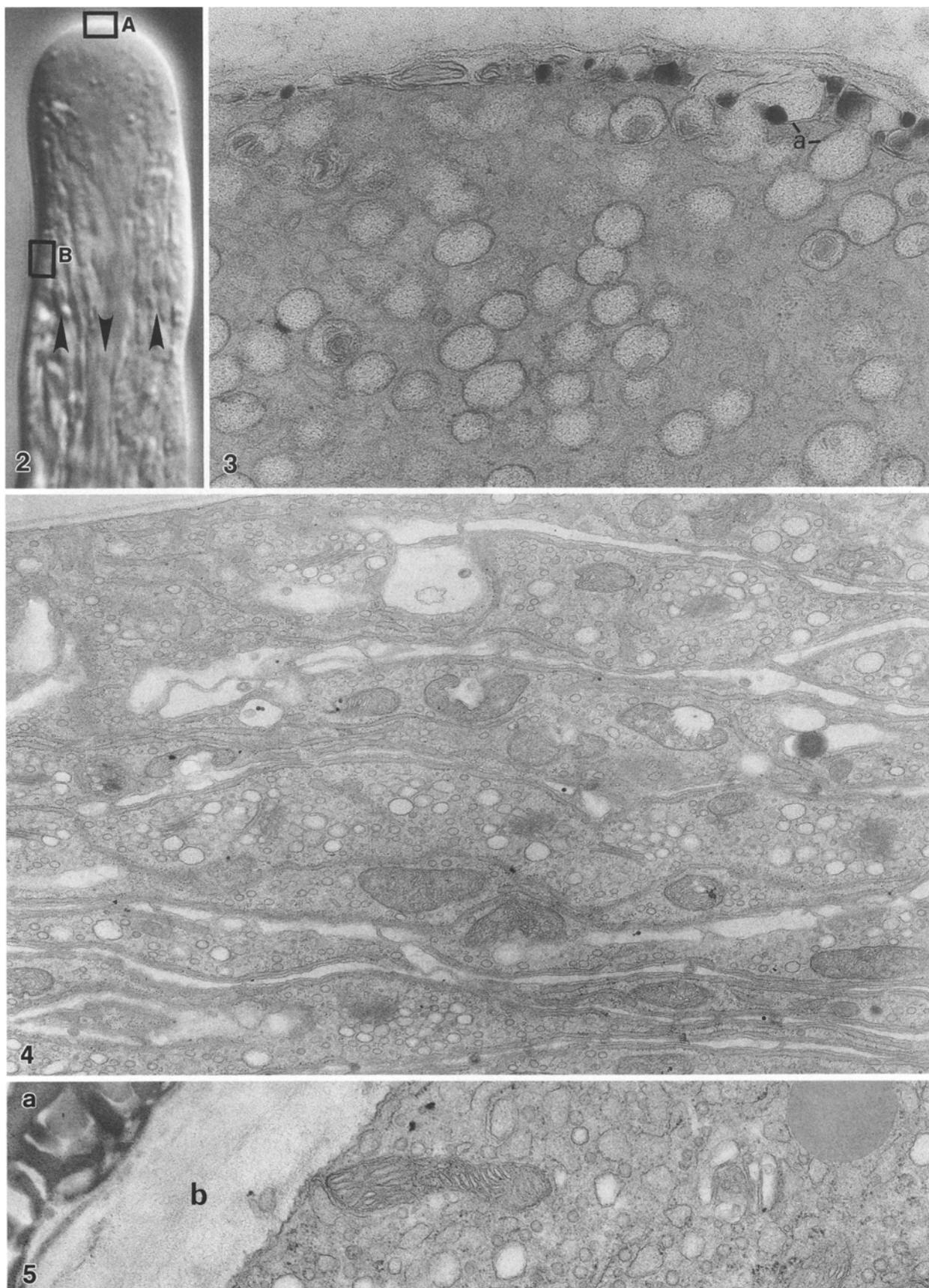


Fig. 2. Apical zone of a living pollen tube, DIC micrograph, 6 s exposure. The arrowheads indicate the directions of flow in the tube in the focal plane of the micrograph. Box A shows the approximate site of the EM of Fig. 3, and box B, that of Fig. 4. Approx. $\times 2400$

Figs. 3–5. EMs of control pollen tubes. **Fig. 3.** Extreme tip region, corresponding in position to box A in Fig. 2. P-particles are seen

apparently in discharge into the the wall (a), which in this region consists of a thin layer mainly composed of microfibrillar pectin. Approx. $\times 36000$. **Fig. 4.** Tube flank in the sub-apical region, corresponding approximately to the position of box B in Fig. 2. Approx. $\times 15000$. **Fig. 5.** Wall and contiguous cytoplasm of a partly vacated pollen grain. The exine with lipid inclusions still remaining in the sexine voids is seen at a, and the intine at b. Approx. $\times 23000$

elles and other inclusions in pollen tubes, simultaneously arresting apical extension. Picton and Steer (1981) showed that the latter response results from the disruption of the tip-growth mechanism and linked this to the likely effect on the actin-based motility system thought to be responsible for delivering the polysaccharide-containing wall-precursor vesicles into the tube apex (review, Steer and Steer 1989). As well as affecting growth and intracellular movement, cytochalasin treatment produces conspicuous changes in the structure of the cytoplasm of the vegetative cell of the pollen tube (Heslop-Harrison and Heslop-Harrison 1989). Continuous video recording of the response of pollen tubes of *Iris* showed that the normal longitudinally striate appearance of the protoplast in the distal part of the tube was progressively destroyed after 80 s exposure to medium containing cytochalasin B at 5 µg ml⁻¹, the cytoplasm eventually aggregating in disorganized masses in which organelle movement was restricted to random local excursions. Concomitantly the tube wall thickened irregularly, initially through the deposition of pectin (Heslop-Harrison and Heslop-Harrison 1989). In this paper we describe some of the fine-structural changes associated with the corresponding responses to cytochalasin treatment in *Endymion non-scriptus*. Standard methods of chemical fixation for electron microscopy have been employed to trace the behaviour of organelles and membrane systems in response to treatment, but we are well aware that these methods do not satisfactorily conserve actin microfilaments in the pollen tube. Effects on the actin cytoskeleton in this species will be described in a further report.

Materials and methods

The observations were made on *Endymion non-scriptus* (L.) Garcke (*Hyacinthoides non-scripta* (L.) Fabr.) from natural populations in west Wales. Pollen was collected from freshly dehiscing anthers and germinated in a medium (GM) containing 1 mM Ca(NO₃)₂, 1 mM H₃BO₃ and 15% sucrose in roller tubes at 22–24°C.

Cytochalasin D (CD) was dissolved in dimethylsulphoxide (DMSO) at 1 mg ml⁻¹, and aliquots added to portions of GM to give a final concentration of 5 µg ml⁻¹. Corresponding concentrations of DMSO alone had no discernible effect on germination or tube growth in controls. Samples of actively extending tubes were collected at 40–50 min by slow-speed centrifugation, transferred to the treatment medium in roller tubes for periods of 15–20 min, and rinsed briefly in fresh GM before inspection and fixation. Earlier experiments showed that pollen tubes subjected to this treatment were usually capable of resuming normal growth after restoration to GM (Heslop-Harrison and Heslop-Harrison 1989).

Samples of living tubes were abstracted at the times required from control and treated cultures and transferred in the same media to glass cells for video recording and streak photography as previously described (Heslop-Harrison and Heslop-Harrison 1989, 1990). The movement of mitochondria was traced using the fluorochrome 3,3'-dihexyloxacarbocyanine iodide (DIOC) at 0.5 µg ml⁻¹ in GM as a vital stain (Johnson et al. 1981). At this concentration DIOC reveals mitochondria as particulate, highly mobile bodies, while membranes of the endoplasmic reticulum are not stained (Fig. 1).

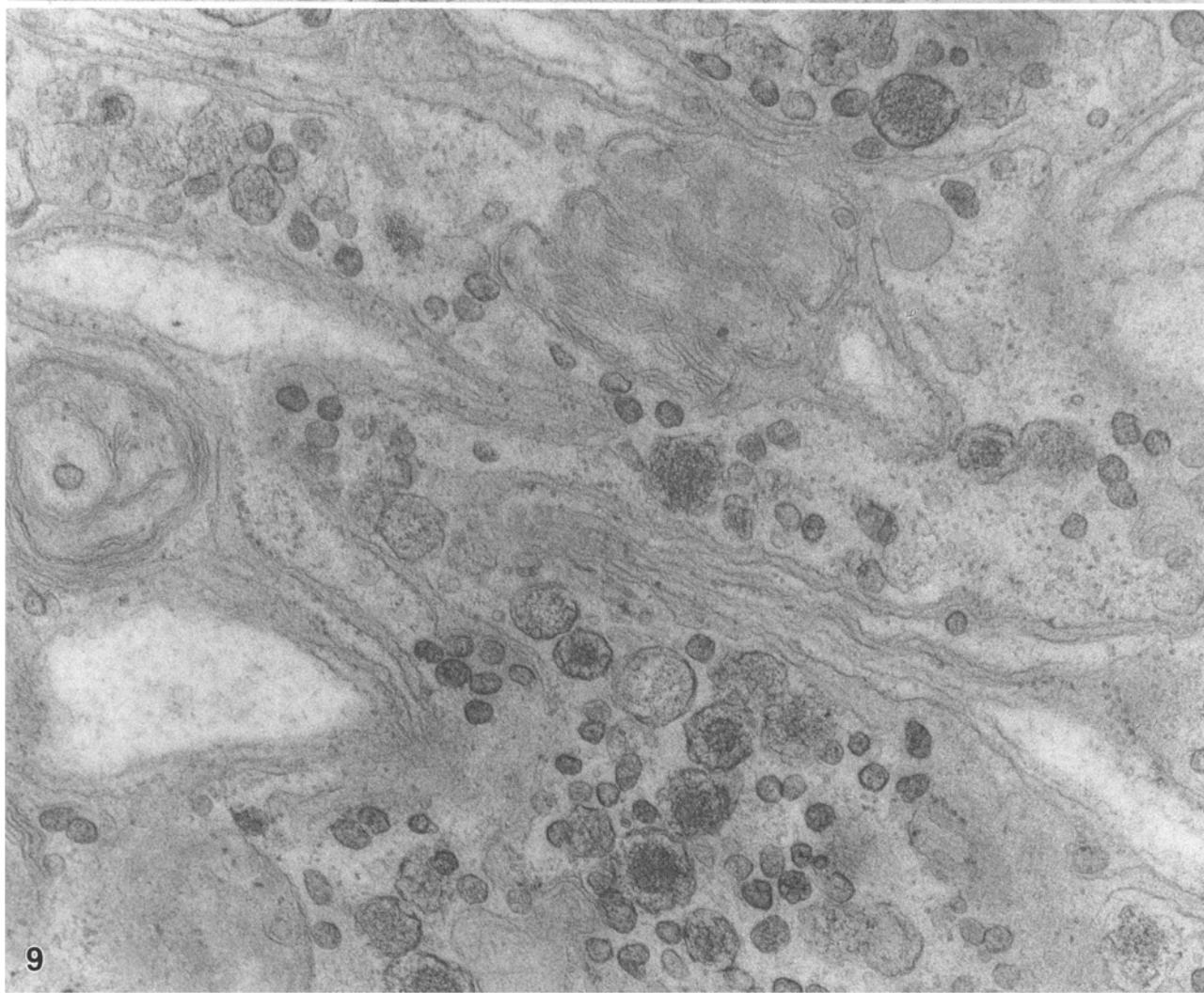
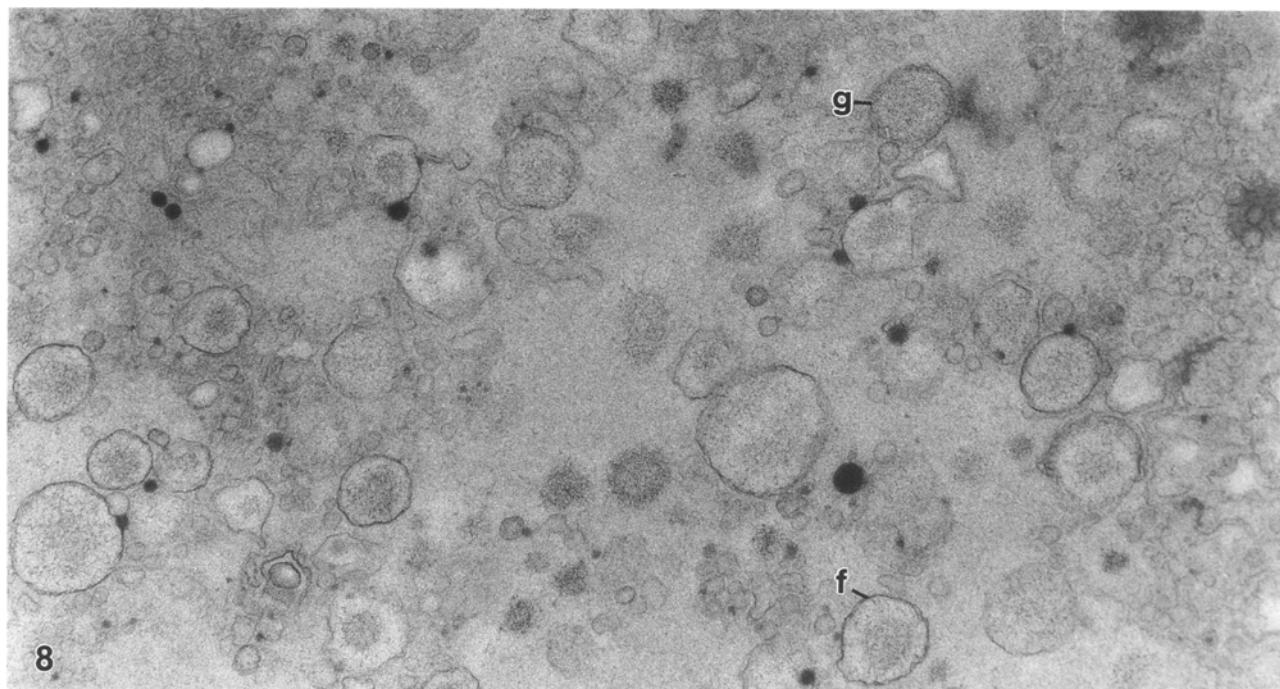
Material for electron microscopy was fixed at room temperature for 3 h in 2.5% glutaraldehyde with 10% sucrose in 0.05 M

phosphate buffer, pH 7.0, and post-fixed in 1% OsO₄ for 1 h at 4°C. The tubes were washed, passed through an alcohol series, and embedded in low-viscosity Transmit resin (TAAB Laboratories, Emmer Green, Reading, U.K.). Thin sections were stained in uranyl acetate and lead citrate by standard methods, or with 1% phosphotungstic acid to give electron density to the polysaccharide content of tube-wall precursor bodies (P-particles; Heslop-Harrison and Heslop-Harrison 1982).

Tubes for optical microscopy were stained as follows: (a) calcofluor white (CFW), 0.001% in 10% sucrose, for cellulosic glucans; (b) alcian blue 8GX, 1% in 3% acetic acid, for the broad class of pectic polysaccharides, and (c) aniline blue, 0.05% decolorised at pH 11 (DAB) in 10% sucrose, for callose. The specificity of these staining procedures for the principal pollen-tube wall components has been discussed in an earlier paper (Heslop-Harrison and Heslop-Harrison 1985), where pertinent references are listed.



Figs. 6, 7. Living pollen tubes in which long-range movement has been inhibited following transfer to medium containing 5 µg ml⁻¹ CD for 15 min. Approx. ×2000. **Fig. 6.** Apical region, DIC micrograph. The tip is already enlarging to form a bulb, and wall thickening has begun (w). The normal apical zonation has been lost (compare Fig. 2), and the organelles and other cytoplasmic inclusions are largely immobilised, although continuous observation of tubes in this state shows that short-range local movements do continue. **Fig. 7A, B.** Distal stretch of the tube behind the apex, a zone in which the longitudinal striae indicative of rapid movement are visible in the normal tube (compare Figs. 2, 4). A DIC micrograph. Continuous observation shows that this characteristic facies develops as the polarised conformation of the cytoplasm in the flow pathways is gradually lost. As Fig. 6 shows, no such conformations are produced in the apical stretch of the tube where there is no extensive membrane system. **B** Fluorescence micrograph, DIOC staining, 8 s exposure. Same field as A, showing the immobilised mitochondria



Figs. 8, 9. EMs of pollen tubes fixed immediately after treatment with CD at $5 \mu\text{g ml}^{-1}$ for 15 min. **Fig. 8.** Largely membrane-free part of a tube in early germination. The P-particles are widely dispersed, and the contents show what are apparently transitional stages between the granular (*g*) and the fibrillar (*f*) state (compare with Fig. 3). Approx. $\times 33000$. **Fig. 9.** Part of a tube in a stretch

comparable to that of Fig. 7. The membrane system and vacuoles no longer show strong longitudinal polarisation. As in Fig. 8, the contents of the P-particles vary in structure, but the numerous smaller, 50- to 60-nm vesicles, (Rosen et al. 1964; Lin et al. 1977) have not been modified by the treatment. Approx. $\times 70000$

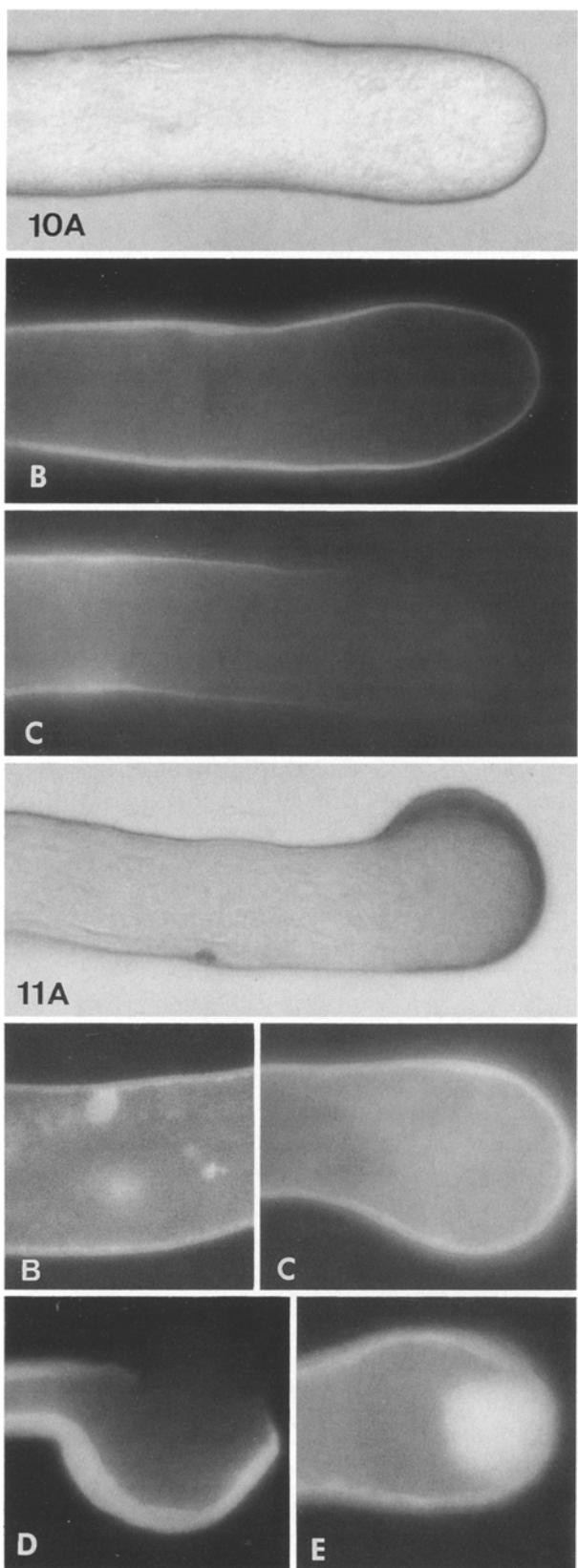


Fig. 10A–C. Apices of unfixed pollen tubes. Approx. $\times 1800$. **A** Bright-field micrograph, alcian blue staining for wall pectin; **B** fluorescence micrograph, CFW staining for cellulosic glucans; **C** fluorescence micrograph, DAB staining for callose. The outer pectic layer invests the whole tube, including the extreme tip. The CFW staining at the apex indicates that some cellulosic material is asso-

Results

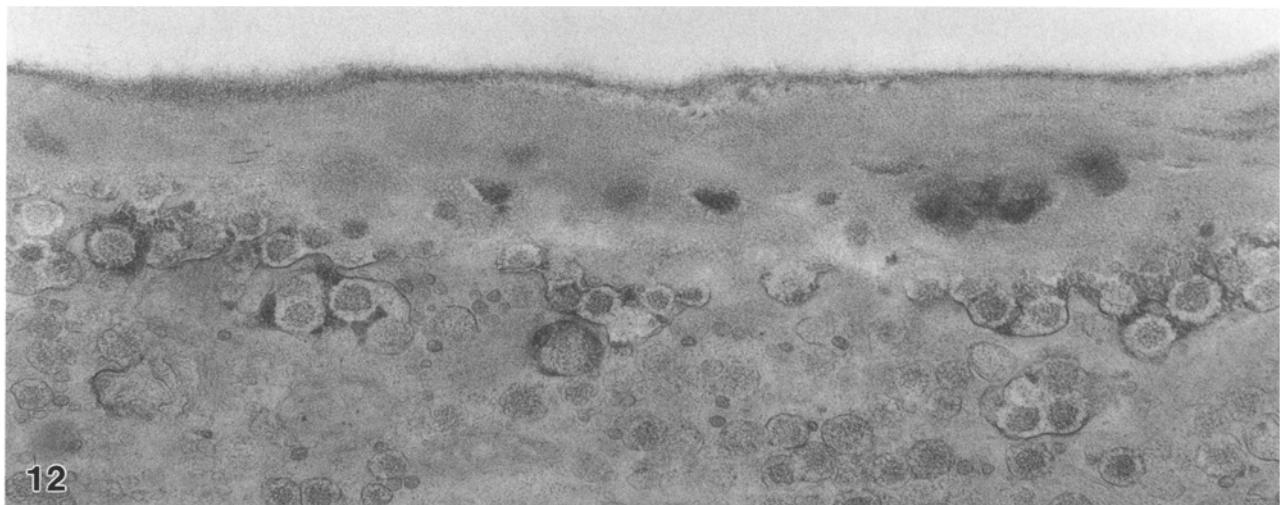
The movement of organelles and other cytoplasmic inclusions in the apices of actively growing pollen tubes of *E. non-scriptus* conforms to the “inverse fountain” pattern general among angiosperms (Figs. 1 and 2; Iwanami 1959; Heslop-Harrison and Heslop-Harrison 1990). Figure 1B, C illustrates the movement of mitochondria in the zone immediately behind the apex. The streak patterns show that they travel rapidly in the main flow pathways, but dwell for periods in the tube tip, where translation from acropetal to basipetal movement takes place, and in islets in the cytoplasm where the flux is temporarily indeterminate.

The EM of Fig. 3, corresponding in position to box A in Fig. 2, shows the characteristic population of polysaccharide-containing P-particles, 250–300 nm in diameter, some evidently fixed during discharge into the wall at the tip. Mitochondria are absent from the extreme tip, but they penetrate into the immediately proximal region (Fig. 1B, C). Figure 4 is from a stretch of a tube corresponding approximately to that outlined in box B of Fig. 2. The orientation of the elongated vacuoles and the endoplasmic reticulum (ER) profiles suggests that the micrograph transects a flow pathway where the various inclusions were in rapid longitudinal movement at the time of fixation. The micrograph illustrates the abundance of active dictyosomes in this sub-apical part of the normal tube. Following 45 min of tube growth the pollen grain is partly evacuated, and the remaining cytoplasm forms a sheath surrounding the central vacuoles. A segment of the pollen grain wall and the contiguous cytoplasm at this stage is seen in Fig. 5. The intine shows no evidence of secondary thickening.

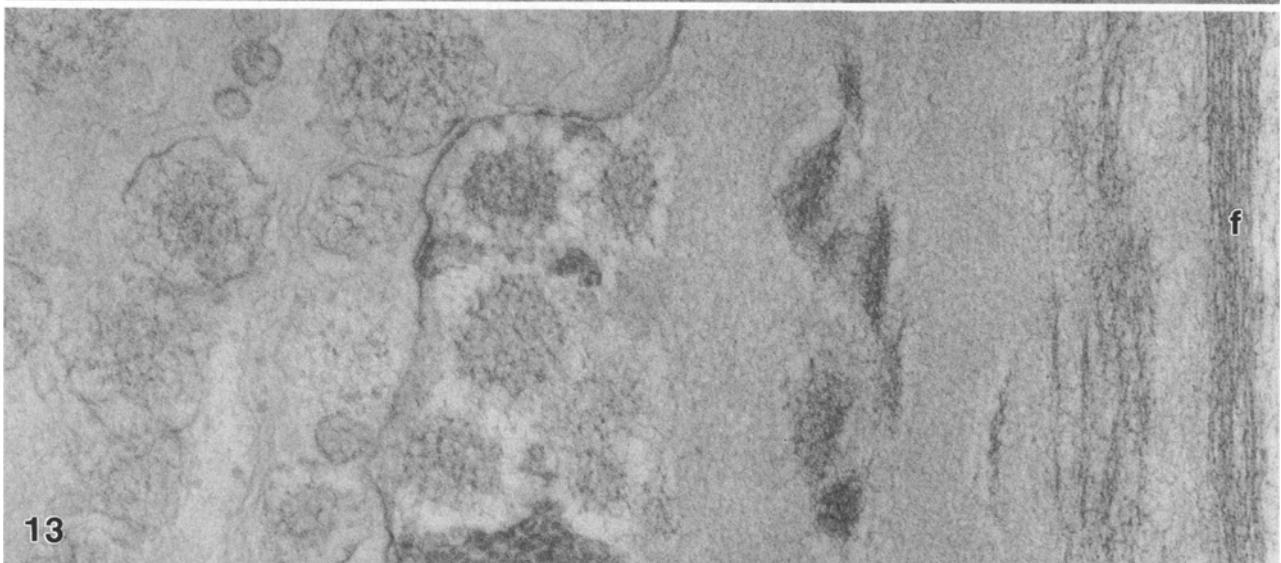
Exposure to CD at $5 \mu\text{g ml}^{-1}$ in GM for 15 min disrupted the apical zonation of the pollen tube, suspended all sustained vectorial traffic, and severely modified the gross structure of the cytoplasm (Figs. 6, 7). Movement was not wholly arrested, however. Wherever P-particles could be identified they showed normal rapid Brownian movement, and continuous video records revealed that organelles and lipid globuli often continued to execute slow random movements with an amplitude

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ciated with the pectin layer. The deposition of callose forming the inner stratum of the tube wall does not begin for some distance behind the apex

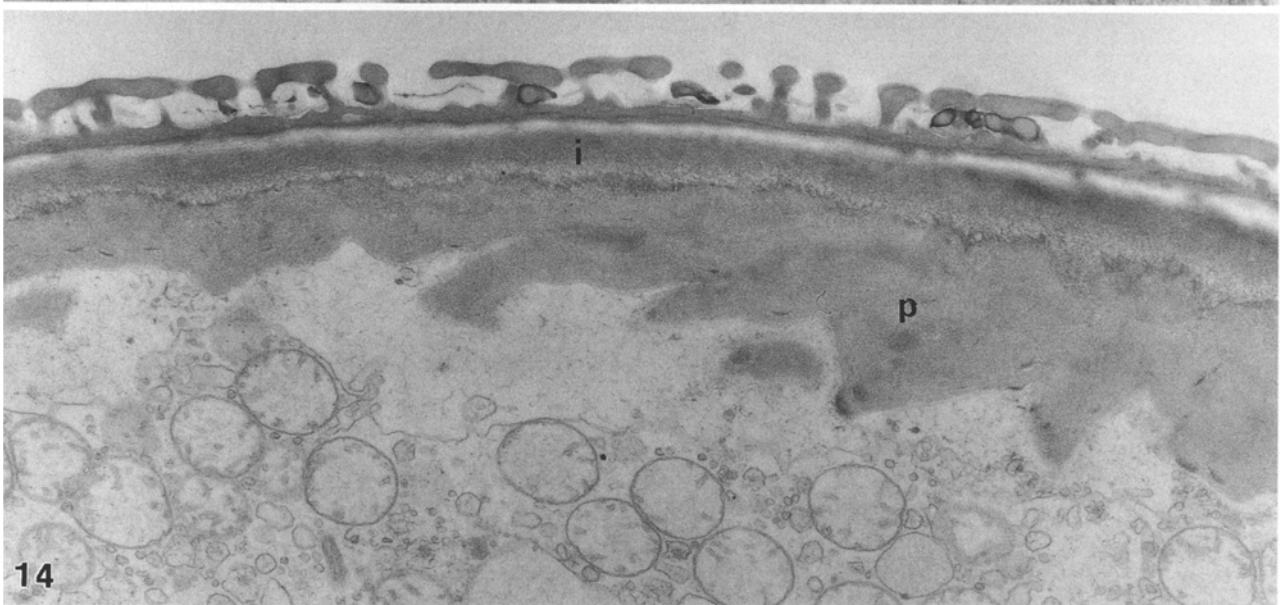
Fig. 11A–E. Unfixed pollen tubes following treatment with CD at $5 \mu\text{g ml}^{-1}$ for 15–20 min. **A** Alcian blue staining. The swollen apex is asymmetrically thickened with pectin, which occurs in irregular masses elsewhere in the tube. Such wall deposits would usually stain also for both classes of wall glucans, with little evidence of stratification. Approx. $\times 1600$ **B** Sub-apical stretch of a tube, CFW staining. The uniform cellulosic thickening of the wall would have been present before the onset of treatment (compare Fig. 10A), and the additional fragmentary masses have accumulated thereafter. Approx. $\times 1800$ **C** Terminal swelling with cellulosic thickening of the wall; CFW staining. Approx. $\times 1600$ **D** Large asymmetric terminal swelling, with heavy callose deposition. Approx. $\times 1200$ **E** Terminal swelling with massive deposit of cellulosic material. Approx. $\times 1600$



12



13



14

Figs. 12–14. EMs of walls from cytochalasin-treated pollen tubes. **Fig. 12.** Longitudinally-sectioned stretch of wall behind the apex. The original tube wall is thickening along its whole length by the random apposition of P-particles, often in partly fused clusters. Approx. $\times 13000$. **Fig. 13.** Detail of the secondary thickening in an apical swelling comparable with that of Fig. 11A. The outer fibrillar layer to the right (*f*) probably represents the original wall of the tube apex; within it are additional irregular zones of partly

incorporated P-particles. The staining reactions of comparable swellings in tubes prepared for optical microscopy reveal their chemical heterogeneity. Approx. $\times 90000$. **Fig. 14.** Radially sectioned wall of an almost empty pollen grain. The intine (*i*) has been heavily thickened by further accumulation mainly of pectic material (*p*). The residual cytoplasm at this stage is attenuated, with swollen mitochondria. Approx. $\times 6500$

exceeding that to be expected from Brownian oscillation alone. The EM of Fig. 8 is from a stretch of a modified tube near to the apex. The P-particles are more widely dispersed than in the normal apex (compare Fig. 3), and they are seen to be structurally heterogeneous, many exhibiting transitional stages with the granular contents giving place to a central core with peripheral fibrils.

The EM of Fig. 9 was from a stretch of a tube modified to the extent seen in Fig. 7A, and comparison with Fig. 4 suggests that the structural change so conspicuous in DIC micrographs of cytochalasin-treated tubes (Heslop-Harrison and Heslop-Harrison 1989) results mainly from the contraction and swelling of the originally elongated vacuoles with concomitant displacement and aggregation of membranes of the ER. Like those in the tube apex, the re-distributed P-particles in the older stretches of the tube exhibit considerable structural variation. On the other hand, Fig. 9 shows that the smaller, 50–60 nm, vesicles of the type known from the distal part of pollen tubes since the earliest observations on the tip-growth system (Rosen et al. 1964) remain unmodified. Active dictyosomes were not observed in any of the treated tubes.

The wall of the normal tube of *E. non-scriptus* shows the usual tripartite stratification (Kroh and Knuiman 1982; Heslop-Harrison 1987), with a thin outer pectic layer overlying and integrating with a cellulosic stratum, which in turn is lined by callose (Fig. 10A–C). Major changes in the wall took place during CD treatment. The first evidence appeared in 10-min samples in the form of abnormal thickening of the pectic layer at the apex. After elongation ceased, most tubes formed a terminal swelling, and in 20-min samples irregular deposits of pectin were present at the apex, along the flanks of the tube (Fig. 11A), and even in the emptying grain itself. The staining reactions indicated that both cellulose and callose were associated with these thickenings (Fig. 11B–D), but no clear stratification could be distinguished with the resolution possible with the light microscope. Isolated pectin accumulations were occasionally observed in the cytoplasm of the vegetative cell remote from the wall, and in some tubes the tips were occluded by massive accumulations of cellulosic glucan (Fig. 11E).

Fine-structural features of the abnormal wall thickenings are seen in the EMs of Figs. 12–14. Figure 12, from the flanks of a tube some distance from the apex, illustrates profiles of apposed and partly embedded P-particles, with apparent transitions to lunulate inclusions which, in turn, are seemingly transformed into more homogeneous fibrillar material on incorporation, much as in the normal process of thickening of the sub-apical part of the tube wall. Figure 13 is of the wall of a terminal bulb comparable with that in Fig. 11D. Several strata are visible, coarsely fibrillar layers alternating with zones of more homogeneous material. A cluster of unincorporated P-particles is present at the cytoplasmic face and another zone of lunulate inclusions, presumably representing partly incorporated P-particles from an early phase of insertion, lies deeper in the wall. The EM of Fig. 14 is of a section transecting the wall of an emptying

pollen grain in which a secondary pectic layer has been deposited within the intine (compare with Fig. 5). The cytoplasm at this time is depauperate, and the residual mitochondria are abnormally inflated.

Discussion

Most of the structural changes in the pollen tube of *E. non-scriptus* produced by CD treatment are explicable if it be accepted that the principal effect of the drug is to corrupt the actomyosin system responsible for maintaining the long-range cyclosis along the tube axis, a pattern of movement which is evidently critically concerned in determining growth polarity in the normal tube and establishing both the associated zonation and the general organisation of the vegetative cell cytoplasm. The principal manifestations are all in conformity with this interpretation. The longitudinal striae visible in distal parts of the living tube clearly reflect polarised movement, and gradual re-distribution of vacuoles and membranes when that movement is blocked would readily account for the conspicuous changes seen in the cytoplasm. Furthermore, the inhibition of movement would necessarily destroy the distinctive inverse-fountain pattern of flow in the apex and eliminate the sorting-out process responsible for maintaining the characteristic pattern of apical zonation (Heslop-Harrison and Heslop-Harrison 1990). By preventing the localised delivery of wall precursors into the extreme tip, these events would disrupt the normal growth system. The subsequent irregular thickening of the tube wall, even into the emptying pollen grain itself, might be expected were the P-particles to be dispersed throughout the tube following the elimination of concerted flow, discharging thereafter into any receptive surface. This would result were they to reach the wall in consequence of essentially random movement. A corollary might be a greater period of retention of the P-particles in the cytoplasm, giving a longer time for the transformation of the contents from the originally granular to the fibrillar state usually achieved during passage into the wall, a transition described in the scheme proposed by Dickinson and Lawson (1975).

If the actomyosin system is a primary target of the cytochalasins in the pollen tube, what then is the basis of its effect? While the bulk of the evidence points to a reorganisation of the actin cytoskeleton in treated cells, there is some uncertainty about the form this takes (Cooper 1987). A recent report by Lancelle and Hepler (1988) based upon observation of cytochalasin-treated pollen tubes of *Nicotiana alata* prepared for electron microscopy by freeze-fixation and freeze-substitution indicated that the principal effect was to eliminate the fine, mainly longitudinally oriented, microfibril bundles characteristic of the normal tube, and to induce the formation of more massive bundles with no particular orientation in relation to the long axis. This report make no specific mention of fragmentation of the microfilament system, an effect that would not in any case be easily detected by electron microscopy. An investigation of the response

of pollen tubes of *Iris* species to cytochalasins B and D using differential interference microscopy coupled with continuous video recording of living cells showed that fibrils forming the pathways of movement of organelles in the older parts of the pollen tube frequently came together to produce more massive columns (Heslop-Harrison and Heslop-Harrison 1989). At least the cores of such fibrils are likely to consist of continuous microfilament bundles, so this response can be reconciled with the observation of Lancelle and Hepler (1988) that cytochalasin treatment led to the appearance of massive microfilament bundles in the tubes of *N. alata*. However, fibrils in the tubes of *Iris* were also seen to break up in cytochalasin-containing media, indicating that in some cases the microfilament bundles were actually fragmented. Currently, the safest conclusion is that the cytochalasins induce both clumping and fragmentation of the actin fibril system in pollen tubes, the latter possibly as a secondary effect of the gross structural changes resulting from the disruption of the normal organisation of the cytoplasm following upon the suspension of long-range axial cyclosis. The fact that organelles and other inclusions continue restricted local movement in the modified cytoplasm, noted also in other cytochalasin-treated plant cells (Palevitz 1980), proves that the motility system is not totally disabled, and may indicate occasional persistence of movement along randomly disposed fragments of the original system.

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