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## Acid tip linear growth in Fungi: requirements for $H^+/Ca^{2+}$ inverse gradients and cytoskeleton integrity

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### Abstract

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The process of apical acidification has been extended from *Neurospora crassa* to the Phycomycetes *Achlya bisexualis* and *Phycomyces blakesleeana* and found to increase according to a gradient inverse to that of  $Ca^{2+}$  ions. Distribution of the latter could be disturbed in germinating conidia of *N. crassa* by anti-calmodulin agents or by ruthenium red and resulted in budding, vesiculated hyphae. The anti-microtubular agent benlate (MBC) allowed outgrowth of abnormal germ tubes from conidia while cytochalasin B presumed to rupture the actin network fully prevented their germination. Our model of proton source and sink for vectorial acidification has been completed and involves a two-step proton gradient: mitochondria  $\rightarrow$  actin "cables"  $\rightarrow$  vesicles functioning as  $H^+$  sinks and thereby energized to drive them through a presumed actomyosin network to the elongating hyphal tips.

### Introduction

Linear growth of germ tubes and hyphae of *Neurospora crassa* has been found to involve an apical acidification of their cylindrical structures (Turian 1979, 1983a). The basifugal protonic gradient detected has been ascribed to a vectorial flow of protons leaked from the apical mitochondria (Turian 1980). Cytochemical evidence was also obtained for an inverse basipetal gradient of  $Ca^{2+}$  (Turian 1979), which led us to suggest that  $Ca^{2+}$  ions were sequestered in mitochondria in exchange of the cytosolic acidifying  $H^+$  ion according to the well-known biochemical scheme (Lehninger 1975, Nuccitelli and Deamer 1983). Parallely, the cytosolic concentration of  $Ca^{2+}$  ions was maintained at a level compatible with the necessary gelation of the microfibrillar network of the apical zone thereby excluding mitochondria from it (Turian 1979).

In this complementary publication, we first extend our description of the  $H^+$  and  $Ca^{2+}$  gradients from the Ascomycete *N. crassa* mentioned above to two major representatives of Phycomycetes presenting both a wide, cylindrical linear growth, the Oomycetous *Achlya bisexualis*, in which an exogenous flow of protons has been electrically

detected (Kropf et al. 1984) and the Zygomycetous *Phycomyces blakesleeenanus* using its outstandingly wide sporangiophore at the 1st elongation stage according to Bergman et al. (1969).

On the assumption of a fundamental interaction between ionic  $H^+$  and  $Ca^{2+}$  inverse fluxes and the apically-oriented cytoskeleton organization, we have investigated the consequences of chemical interferences with  $Ca^{2+}$ -transport and with both microtubular and microfibrillar organizations. From the apparent need for integrity of both of these structures, we propose a scheme interrelating them with the vectorial transport of protons from their source to their sink along a gradient suggested to act as the directional driving force for the vesicles involved in the elongation growth of fungal hyphae.

### Material and methods

*N. crassa*, wild type strain Lindegren STA<sub>4</sub> 262 A, has been maintained and subcultivated on synthetic Vogel minimum medium (Vogel 1956). Macroconidia harvested in sterile distilled water from aerial hyphae have been inoculated on the same synthetic medium which normally includes 0.68 mM of  $CaCl_2$  (Vogel 1956).

The pH tests were realized using acridine orange as fluorescent probe, the normal green fluorescence (530 nm) being quenched by acidity to a less intense fluorescence (yellowish-orange to red fluorescence (660 nm)). As for calcium levels, they were microscopically evaluated using as stain a saturated solution of alizarin yellow S in distilled water as already described (Turian 1983 a).

The anti-calmodulin agents, chlorpromazine and penfluridol have been dissolved in water and dimethylsulfoxide (DMSO) 0.1% and conidia were germinated in the presence of the inhibitors to the final concentrations of 86 and 6.4  $\mu M$  respectively. As inhibitor of  $Ca^{2+}$  transport into mitochondria, ruthenium red (RuR) has been sterily incorporated into the liquid or solid agar (2%) synthetic medium.

For tests with benlate (MBC = methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate) conidia were germinated at 25 °C on glass slides, over wet filter paper in Petri dishes, in a drop of liquid Vogel's medium added with one drop of  $5.2 \times 10^{-6}$  M benlate. The toxic agent was first dissolved in DMSO and then diluted with distilled water to a final concentration of 1.0  $\mu g/ml$  ( $5.2 \times 10^{-6}$  M MBC) and 0.1% DMSO. By mixing both liquid drops, we could assume that our germination solution contained roughly  $2.5 \times 10^{-6}$  M MBC and 0.05% DMSO (separately controlled to have no morphogenetic effect by itself).

Cytochalasin B (CB) was dissolved in DMSO and diluted to a final concentration of 0.1% DMSO. The concentration of CB used in this study varied from 5 to 200  $\mu g/ml$ . Tritiated cytochalasin B, [ $^3H$ ]CB, specific activity 17 Ci/mM was used. A stock solution containing [ $^3H$ ]CB (5  $\mu g/ml$ ) in 0.1% DMSO was diluted with Vogel's medium containing cold CB to give a final concentration of 60  $\mu g/ml$ . After 2 h at 30 °C, pregerminated conidia in normal Vogel's medium were incubated for 2 h in the [ $^3H$ ]CB-Vogel's medium, then they were fixed for electron microscopy according to Ton-That and Turian (1978).

After digestion with 2% helicase in Vogel's medium during 30 min at 37 °C, cytoplasmic extraction of germlings by glycerination was performed according to Ishikawa et al. (1969).

HMM subfragment-1 (S-1) was added to the extract preparations from 3 h at 30 °C germlings in Vogel's medium (Allen and Sussman 1978) at a final concentration of 0.2 mg/ml. Negative staining of microfilaments was prepared according to Woolley (1972).

## Results and discussion

### $H^+$ / $Ca^{2+}$ apical gradients

In *Achlya bisexualis* and *Phycomyces blakesleeanus*, both gradients have been detected simultaneously by observation of the intensity of quenching of the fluorescent probe acridine orange expressing the degree of cytoplasmic protonization and of the intensity of the reddening for  $Ca^{2+}$  using the stain alizarin yellow S.

In the narrowing apices of the *Achlya* hyphae, quenching of acridine orange was microscopically visualized to dark copper with a red tinge difficult to register photographically (Plate 1 d). The acidified, triangle-shaped tip was found to correspond to the most yellowish zone – lowest in  $Ca^{2+}$  – observed with the alizarin reagent which stained in red – high  $Ca^{2+}$  – the subapical, mitochondria-rich zone (Plate 1 e). To insure that such staining disjunction, inverse to pH gradient, was not related to a lethal condition of the hyphae, we let a few hyphal tips resume their elongation growth following a dilution of the alizarin solution to a more vital concentration; we could observe the maintenance of the yellow tinge in the homogeneous tip triangle (Plate 1 e) contrasting with the pinkish red staining of the moving, long subapical mitochondria. A similar sharply contrasted staining by alizarin has been observed in outgrowing conidia of *Monilia fructigena*, the transition to the red  $Ca^{2+}$ -rich subapical zone being especially noticeable along the axis of emergent germ tubes (Plate 1 c).

Fluorescence quenching of acridine orange to a vivid orange tinge has also been observed in sporangiophores of *Phycomyces* at various stages of their apical elongation (5–15 mm high). Quenching to red was only observed in the fringe of the ultimate tip (Plate 1 f), a subzone which should be deprived of ribosomes (by analogy with other hyphal apices, see Grove 1978, Turian et al. 1985) and found to give a relatively more intense Na-nitroprussiate positive – pink – reaction for -SH groups (unpublished observations). A yellowish quenching is prolonged backwards along the cortical zone of the side wall of the sporangiophore tip surrounding the extremity of the central vacuole.

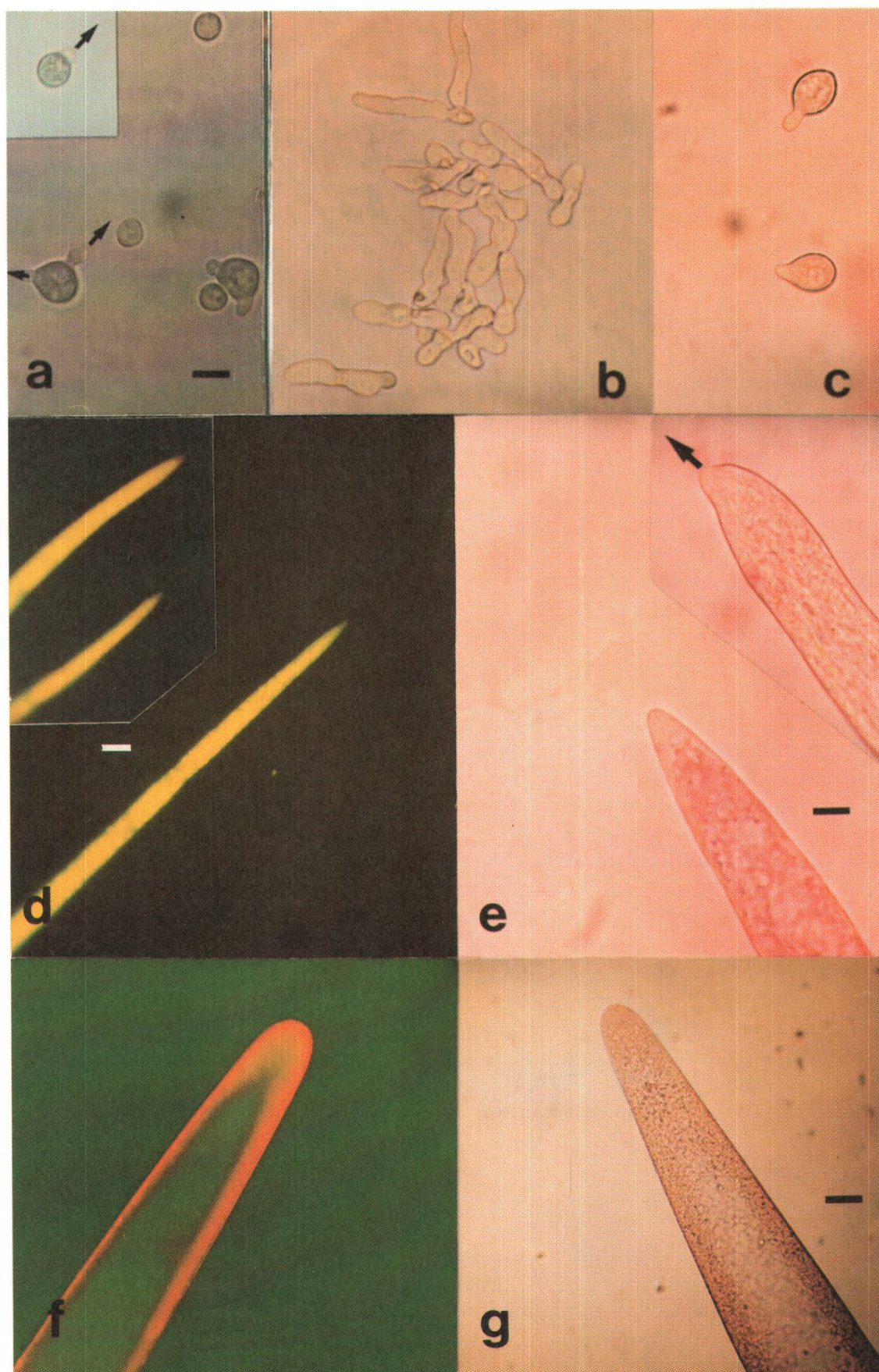
The parallel staining of the sporangiophores with alizarin yellow S allowed to observe a progressive basipetal reddening leaving a yellowish – low calcium – tip zone (Plate 1 g). Such apparently less sharp gradiential distribution of  $Ca^{2+}$  along the *Phycomyces* sporangiophores could be related to the aerial elongation:  $Ca^{2+}$  ions would not be pumped from the tips to the mitochondria as in hyphae grown in liquid, calcium containing, media (*Neurospora*, *Monilia*, *Achlya*) but rather would be translocated by ionic fluxes from the basal cell embedded in the nutrient solid medium. By comparison, germ tubes outgrowing from pollen grains incubated in liquid medium had shown yellow tips in alizarin (Turian 1981) and recent X-rays probing has confirmed their lower  $Ca^{2+}$  tip content compared to more backward zones (Reiss et al. 1985).

### $Ca^{2+}$ -effectors

#### Anti-calmodulin agents

Calmodulin is a calcium-binding protein acting as one of the principal intracellular calcium receptors in eukaryotic cells. It has been isolated from *N. crassa* (Ortega Perez et al. 1981). If, as expected, it regulates  $Ca^{2+}$ -depending activities in the outgrowing elongating hyphae, interference with it using anti-calmodulin agents should bear on these morphogenetic processes.







We have tested two phenothiazines, chlorpromazine and penfluridol, added at micromolar levels to the Vogel's medium: both have delayed germination of conidia of *N. crassa*. The outgrown tubes, frequently two, were enlarged, irregular in shape and often prematurely septated. Stained in alizarin yellow S they did not exhibit a conspicuous color gradient while showing refringent yellowish inclusions (Plate 1 b).

On the basis of this observed levelling of the  $\text{Ca}^{2+}$ -gradient in abnormally germinated conidia, the anti-calmodulin treatment could produce a disturbance of the homeostatic regulation of the  $\text{Ca}^{2+}$ -level required by the directional organization of the cytoskeleton meshwork. The disturbance of this organization is more visibly expressed at the ultrastructural level by an apparent homogenized distribution of the organellar system (Ortega Perez, unpublished results, 1983).

### Inhibitors of $\text{Ca}^{2+}$ -transport

Ruthenium red (RuR) is a standard inhibitor of  $\text{Ca}^{2+}$  transport into mitochondria (Moore 1967, Bygrave et al. 1978). In preventing  $\text{Ca}^{2+}$ -sequestration in these organelles, RuR is expected to prevent the reciprocal efflux of  $2 \text{ H}^+$  normally contributing to the cytosolic acidification while maintaining higher and disturbing level of  $\text{Ca}^{2+}$  around the cytoskeletal components. In fact, RuR could drastically modify the cylindrical shape of the hyphae outgrown from conidia *N. crassa* germinated in its presence.

Conidia of *N. crassa* inoculated in solid Vogel's medium normally grow out after 2 h at  $25^\circ\text{C}$  as a single germ tube which then elongates into a linearly growing hypha. When conidia were inoculated on plates of the same synthetic medium containing ruthenium red, outgrowth of their generally single germ tube was delayed (3–4 h at  $25^\circ\text{C}$ ). With 5 mM RuR, linear growth of still apparently cylindrical hyphae was reduced by close to 50%. In the presence of 10 mM, RuR colonial mycelia developed very slowly by repeated budding of highly vesiculated hyphae (Fig. 1).

Conidia sedimented at the bottom of the liquid synthetic medium containing 20 mM of RuR could still germinate but very abnormally (Fig. 1 insert), also by basifugal budding of vesiculate and highly vacuolate yeast-like cells. However, few spherical abnormally germinated conidia could secondarily escape the morphogenetic effect and after a few vesiculations could recover the partially cylindrical shape of normal hyphae (Fig. 1 c).

### Plate 1.

Gradients of protons revealed by quenching of acridine orange ( $10^{-1}$  mM) to a dark copper tinge in the hyphal tips of *Achlya bisexualis* (d), to red (extreme tip) turning to yellow (vacuole top side) in the apex of *Phycomyces blakesleeianus* sporangiophore (f).

Parallel staining with alizarin yellow S for gradients of  $\text{Ca}^{2+}$  ion in the same species, respectively (e, g). Pale yellow staining with alizarin – low  $\text{Ca}^{2+}$  level – in a regenerating tip of *Achlya* (arrow on insert e). Idem in the germ tubes outgrowing from conidia of *Monilia fructigena* (c). Fading of the alizarin staining – and the calcium gradient – in chlorpromazine-treated and abnormally germinated conidia of *N. crassa* (b). Bipolarly germinated (arrows) conidia of *N. crassa* in the presence of benlate (a); buds devoided of conspicuous pH gradient when stained in bromocresol green compared with control germinated conidia (insert with arrow on yellow germ tube).

Color photomicrographs on film Fujicolor-400 (a, b, c, e, g) and Ektachrom film for fluorescent pictures (d, f) on Leitz Dialux 20 epifluorescent (d) and Olympus microscopes (fluorescence-excitation light through 400 nm filters, emitted light through a 500 nm-broad bandpass filter).

Bars = 10  $\mu\text{m}$  (50  $\mu\text{m}$  for *Phycomyces* (f, g) and *Achlya* (d)).

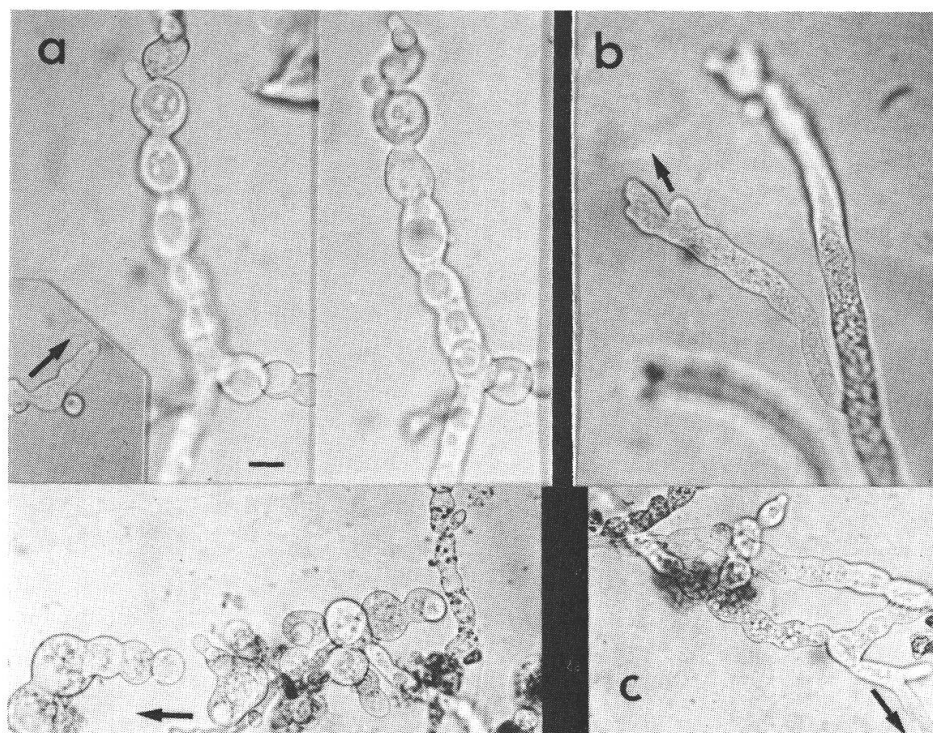


Fig. 1. Colonial type of growth of *Neurospora crassa* on solid synthetic medium containing ruthenium red: a) 10 mM, 2 days at 25 °C, double focus on budding and vesiculated hyphae; insert, slowly germinating conidium (arrow) after 7 h of incubation in liquid medium (20 mM RuR); b) idem, with widened, short, ramified hypha (arrow); c) 5 mM, 2 days at 25 °C, a few recovering normally elongating hyphae (arrows) among vesiculated structures. – Photomicrographs on Ilford Pan F film with Wild M-20 microscope. Bar = 10  $\mu$ .

## Cytoskeleton

### Anti-microtubular effects

Destruction of the ordered arrangement of organelles along cellular axes upon colchicine or benlate (MBC) treatments might result from interference with microtubules functions (Dustin 1978). In fungi, multiple emergence of branched germ tubes was observed on conidia of *Botrytis cinerea* treated with benomyl (Richmond 1975); this effect was considered a consequence of microtubules destruction as confirmed by Howard and Aist (1977) in apices of hyphae of *Fusarium acuminatum* treated with MBC, the hydrolytically produced toxic moiety of benomyl.

We have extended that observation of the multigerm tube effect of MBC to conidia of *N. crassa* in focussing our interest on the shape of the outgrown germ tubes. We observed that, in the presence of  $2.5 \times 10^{-6}$  M benlate (MBC), most of the conidia which could germinate after 3 h at 25 °C, grew out into two inflating germ tubes which could uniformly be stained with bromocresol green, a pH indicator which switches to its

acidic yellow tinge in the control germ tubes (Plate 1 a). When further incubated, the inflated germ tubes became undulated as also noticed with *Fusarium* hyphae (Howard and Aist 1977, 1980).

#### Anti-microfibrillar effects

Experiments have been focused on the germination outgrowth as the first organizational stage for linear growth.

Cytochalasin B (CB), the well-known inhibitor of actin polymerization (Fulton 1984) very effectively prevented the polarizing stage in conidia of *N. crassa* dispersed in liquid Vogel's medium. In the presence of 60 µg/ml, about 10% of the conidia presented short and wide germ tubes when CB was added to pregerminated conidia (2 h at 30 °C in Vogel's medium). Linear growth of their tubes was stopped and accompanied by a thickening of their wall (Plate 2, a and b).

On preliminarily obtained thin-sections, it could be checked that organelles were uniformly distributed, an effect comparable to that obtained with anti-microtubular agents. This is not unexpected because microfibrils are closely connected with microtubules serving as guiding tracks. In fact, it has recently been shown that stabilization of microtubules with N<sup>6</sup>,O<sup>2</sup>-dibutyryl-adenosine 3':5'-cyclic monophosphate has led to a protection of the actin microfibrillar network against the disorganizing effects of cytochalasin in *Mucor globosus* with parallel prevention of the widening of the apices (Ton-That and Hoch 1984).

Assays with tritiated cytochalasin (<sup>3</sup>H-CB) have shown a generalized incorporation appearing to be located in lipid globules, some mitochondria and in the peripheral areas below the plasma membrane, presumably on the polymerized actin located in the cortical zone of the bulging germ tube (Plate 2 b).

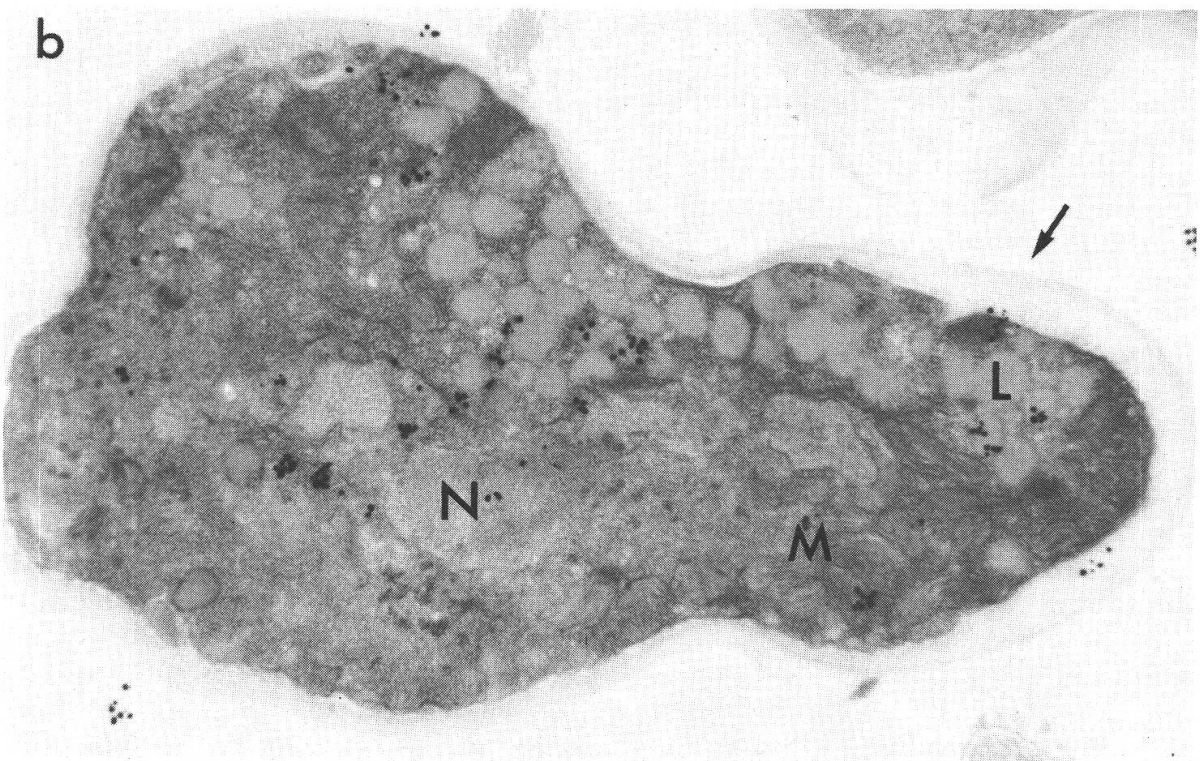
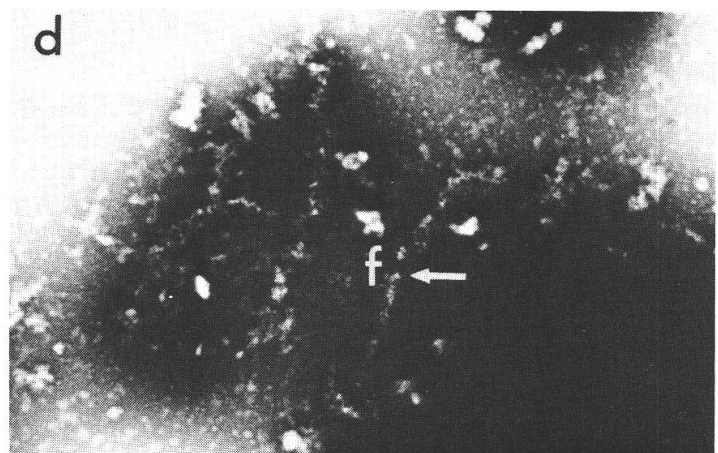
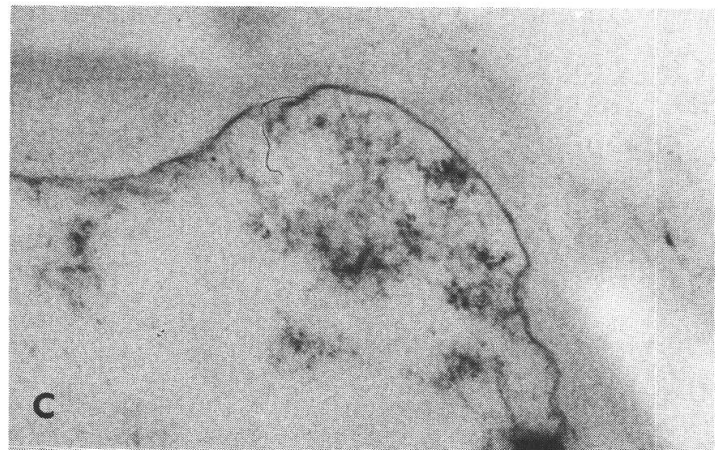
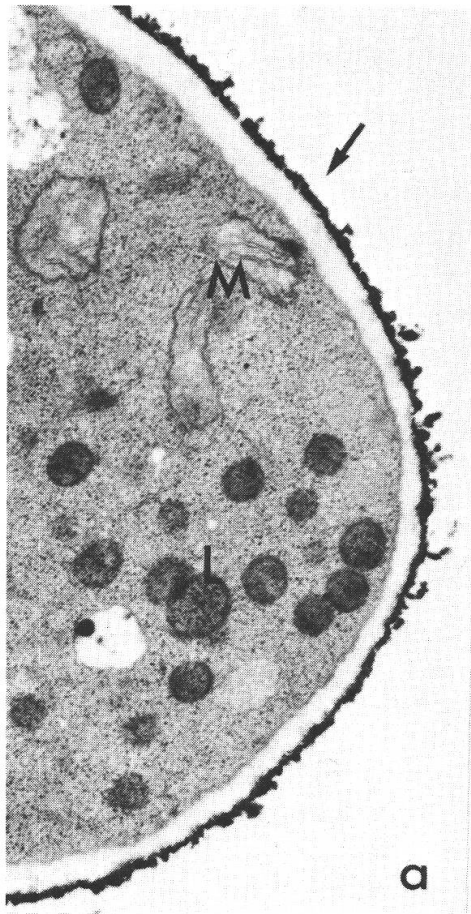
Following glycerol extraction of the cytoplasm of early germinating conidia (3 h, 25 °C) according to Ishikawa et al. (1969), we observed a bulging of the plasma membrane over a meshwork of microfibrils presumably at the outgrowth site of the germ tube (Plate 2 c).

In applying myosin subfragment-1 (extracted, purified from muscle) on extracts of *N. crassa* germlings, we could detect by negative staining, the arrowheads indicatives of the actin nature of microfilaments (Plate 2 d).

#### Proposal for an integrative scheme

Preceding experiments based on the use of respiratory inhibitors (Turian 1980) and uncoupling agents (Turian and Michéa-Hamzehpour 1983) have led us to suggest that the apical acidity first detected in *Neurospora* germ tubes and here extended to additional species, is due to protons extruded from mitochondria but prevented to circulate back to them – according to Mitchell's chemiosmotic theory – by their vectorial diversion to a sink. Such a proton sink was proposed to originate from the frontal contact of a few mitochondria from a random cluster (Turian 1985) with a plasmalemmal site through a patch of electron-dense material (Turian and Geissler 1984) suspected to induce respiratory uncoupling. Following our findings of actin-like microfibrillar material below the plasmalemma (Plate 2 c), we are now inclined to think that protons could be directed through actin microfibrils to the thereby depolarized plasmalemmal site of outgrowth, while providing by local acidity and low Ca<sup>2+</sup> the conditions required for





the increased protein crosslinking (Fulton 1984) of the microfibrillar patches. These latter, by their expansion, would force backward the frontal mitochondria by the cytosol-gel sorting out process previously described (Turian 1979) as creator of the well-known exclusion zone of the hyphal apices. That primary sink of protons provided by the microfibrillar network of the expanding tip of the germ tube must be necessarily relayed by the exocytic microvesicles on their way to the tip.

In our effort to explain the vectorial protonation of the apical cytoplasm, we had to take into account the fact that the protons ejected from mitochondria cannot penetrate far into the water bulk phase (Skulachev 1981, p. 10), and we have been in search of protons-conducting specific proteins possibly acting by a concerted proton transfer. It could well be actin which, being electrically polarizable as proposed by Tirosh et al. (1980) for motile systems could function by the so-called tunneling of protons (Lehninger 1975); this process would implicate  $H^+$  conduction by jumping along the hydrogen-bounded lattice of  $H_2O$  molecules, the last water molecule becoming a hydronium ion,  $H_3O^+$ . As expected, any loss of integrity of the hydrated actin "cables", as produced by cytochalasins, leads to prevention of germ tube outgrowth, contrarily to the microtubules stringently requested only for hyphal elongation.

Continuity between the cytoskeleton and the surface of mitochondria or of cytoplasmic vesicles (for fungal microvesicles, see Hoch and Howard 1980) has been observed in many types of cells (Alberts et al. 1983, Dillon 1981). When they come to contact the exocytic vesicles, actin "cables" could provide them with the high "debit" of protons required for the activity of a presumed membranal  $H^+$ -ATPase ensuring the gradiential accumulation of protons into vesicles. This uptake of protons into vesicles functioning as a major  $H^+$  sink could involve cotransport of wall precursors such as N-acetyl-glucosamine by analogy with what occurs in the so-called amine-trapping vesicles. The acidification of the apically migrating vesicles could possibly be relayed and amplified by the exogenous cotransport of  $H^+$  with glucose and/or amino acids into hyphae of *Achlya* elongating in nutrient-rich liquid media as recently demonstrated by Harold's group (Kropf et al. 1984).

If, as is expected on chemiosmotic grounds and quite recently found by Henry et al. (1985), the energy of the proton gradient in the chromaffin vesicles could be reverted to synthesize ATP on their membrane surface, the newly available ATP could be used by an actin-activated myosin ATPase; following our implication of an actomyosin system in the apical expansion of fungal hyphae (Turian 1983 b), we could then visualize myosin fibrils attached to the vesicles on one hand and to actin on the other, to provoke vesicular migration by a shearing process as proposed by Williamson (1976) for algal chloroplasts.

#### Plate 2.

Conidia of *Neurospora crassa* pregerminated 2 h at 30 °C in Vogel's medium: a) Part of conidium after 2 h treatment with 60 µg CB/ml. Note the thick wall with irregular dense deposits (arrow). 20,000×. b) Autoradiograph of a section through a germinated conidium after 2 h treatment with [ $^3H$ -CB] (5 µg/ml). Many grains are present over the lipid granules, in some mitochondria and next to the plasma membrane. Note the thick wall of the germ tube (arrow). 16,000×. c) Germinated conidia 3 h at 25 °C in Vogel's medium. 2 days *in situ* glycerinated conidium. Microfilaments are located at the presumptive site of outgrowth. 32,000×. d) Negatively stained preparation of the HMM- $S_1$  treated homogenate. Thin-filaments (f) are bound with HMM- $S_1$  and show the arrowhead structures. 40,000×. L (lipids), M (mitochondria), N (nuclei).

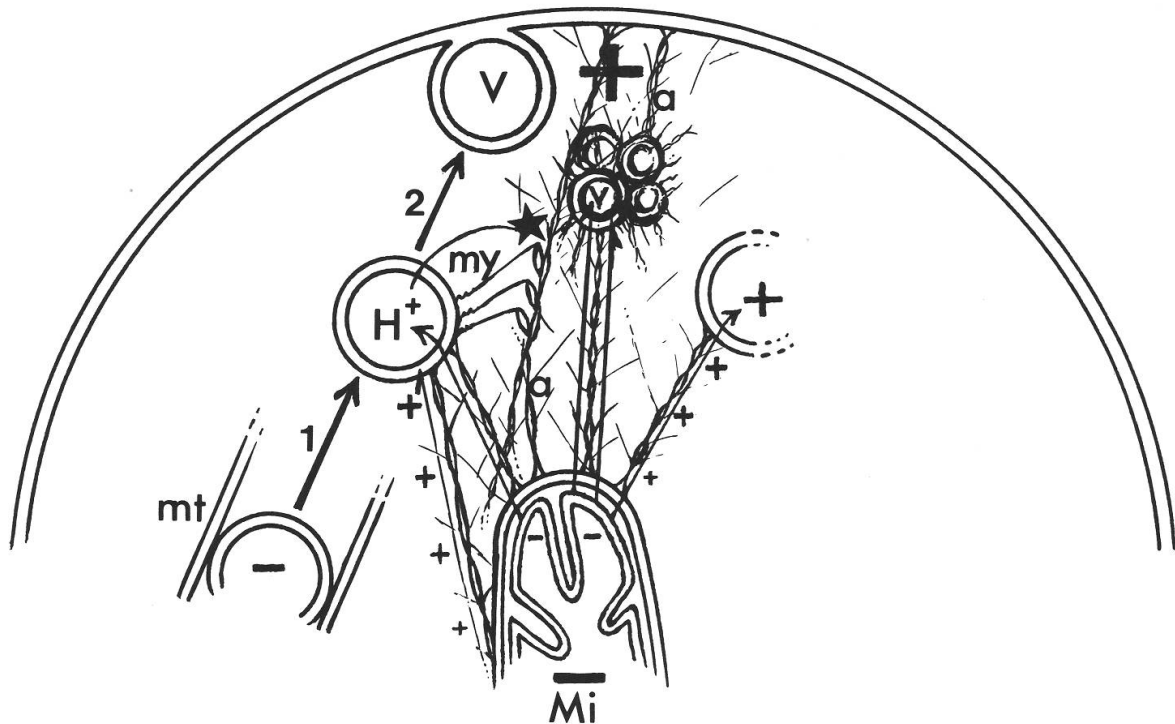


Fig. 2. Hypothetical circuitry of vectorial protonation in a germ tube outgrowing from the conidium model of *Neurospora crassa*: mitochondrial (Mi) source of protons conducted by electrically polarized and helicoidally polymerized microfibrils of actin (a) to the vesicles (V) functioning as main  $H^+$  sink aside of the actin-coated microvesicles (v) or filasomes aggregated into the acidic "Spitzenkörper". Following their first move (1) by self-electrophoresis, vesicles are further apically directed (2) by conformational changes of myosin (my) hooked both on them and actin microfibrils; reverse synthesis of ATP (\*) for actin-activated myosin ATPase would insure self-entrainment of the "protonic motor".

Finally, the proton motive force ( $\Delta\mu H^+$ ) vectorially directed in a two-step process from the mitochondria through actin fibrils to the vesicles could be somehow transduced into an electro-mechanical driving force for the vesicular traffic to the expanding hyphal tip. As proposed by Skulachev and his group for other types of motile systems (1981), linear growth of fungal hyphae would thus be vectorially powered by a "protonic motor" (integrative model, Fig. 2).

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## Résumé

L'acidification apicale a été généralisée du *Neurospora crassa* aux Phycomycètes *Achlya bisexualis* et *Phycomyces blakesleeana*; elle s'accroît selon un gradient inverse de celui des ions  $Ca^{2+}$ . Cette répartition a pu être perturbée dans les conidies en germination de *N. crassa* par des agents anti-calmoduline ou par le rouge de ruthénium, avec



pour résultat le bourgeonnement d'hyphes vésiculeux. L'agent anti-microtubules benlate (MBC) a permis l'émergence de tubes germinatifs anormaux à partir des conidies alors que la cytochalasine B, présumée capable de rompre le réseau d'actine, a complètement bloqué leur germination. Notre modèle de source et écoulement de protons rendant compte de l'acidification vectorielle a été complété comme impliquant un gradient protonique en 2 étapes: mitochondries → "câbles" d'actine → vésicules fonctionnant pour la captation des ions  $H^+$  et, par ce mécanisme, énergisées pour leur conduite, au travers d'un réseau actomyosinique présumé vers les extrémités des hyphes en élongation.

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