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Hyphal Tips of Wild-Type and Spreading Colonial Mutants of *Neurospora crassa*

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Abstract. The ultrastructure of the hyphal tips of wild type and spreading colonial mutants (spco) of *Neurospora crassa* was studied. The results suggest that the observed concentration of vesicles in the hyphal tips of wild type, cot 3, spco 1 and spco 12 is a consequence of the fact that in each strain the number of vesicles *per unit length* of the long axis the hypha remains approximately constant. Spco 9 differed from the other strains in that a proportionally longer region of its hyphal tips contained a high concentration of vesicles.

There was a direct relationship between the length of the tapered region of the hyphal tips of the various strains and their extension rates; the tapered region of a tip probably represents the extension zone of the hypha and varied in length from about 2 μm (spco 12) to about 33 μm (wild-type).

Key words: *Neurospora crassa* — Growth — Extension Zone — Vesicles — Tips/Hyphae — Spitzenkörper.

Although it is well established that hyphae increase in length by apical extension there is little information about the precise region of the hyphal tip which is extensible. The tall conidiophores of *Aspergillus giganteus* have an apical extension zone of about 200 μm (Trinci and Banbury, 1967, 1968) whilst stage IV b sporangiophores of *Phycomyces blakesleeanus* have an intercalary extension zone of about 3 mm (Ingold, 1962). Unfortunately it is not easy to apply the techniques used to determine the extension zone of these aerial hyphae to studies of mycelial hyphae.

Several workers have demonstrated the presence of small vesicles in the tips of fungal hyphae (Grove and Bracker, 1970; Bartnicki-Garcia, 1973). The vesicles are thought to contain precursors of cell wall polymers and/or the enzymes required for the synthesis of these polymers and their incorporation into the existing wall. The vesicles also supply membrane which is added to the protoplasmic membrane at the hyphal apex, enabling it to expand rapidly in surface area.

The present investigation was undertaken to obtain estimates of the length of the extension zone of mycelial hyphae of wild type and spreading colonial mutants (spco) of *Neurospora crassa*. Ultrastructural studies

were also made to investigate the relationship between the rate of hyphal extension and the apical distribution of vesicles. The spreading colonial mutants of *N. crassa* were chosen for this study because although they have the same maximum specific growth rate as the wild type their maximum rate of hyphal extension varies from 74 to over 1000 $\mu m\ h^{-1}$ (Trinci, 1973).

Materials and Methods

Organisms and Medium. The origin of *Neurospora crassa* SYR-17-3A (wild type), spco 1, 3, 9 and 12 has been described previously (Trinci, 1973). Cot 3 is a temperature sensitive mutant which has wild-type characteristics at 25°C. The strains were grown in 9 cm plastic Petri dishes containing 20 ml of Vogel's minimum medium (Vogel, 1956) with 1% (w/v) sucrose as the carbon source. Cultures were grown at 25°C.

Electron-Microscopy. Colonies were fixed at 25°C for 1 h by flooding plates with 3 or 4% (v/v) glutaraldehyde in 0.05 M cacodylate buffer or 0.1 M phosphate buffer at pH 7.2. Sectors were cut from the margins of the colonies and left overnight at 4°C in buffered glutaraldehyde. The sectors were washed thoroughly in several changes of distilled water over a period of about 2 h and then post-fixed in 1% (w/v) aqueous osmium tetroxide at room temperature (about 18°C) for 1 h. After they had been thoroughly washed in distilled water some sectors were stained for 1 h in 0.5% (w/v) aqueous uranyl acetate. After dehydration the material was embedded as described previously (Trinci and Collinge, 1973). Serial longitudinal and transverse sections were cut of leading hyphae. Sections were stained with 2% (w/v) aqueous uranyl acetate or 50% (v/v) aqueous ethanol saturated with uranyl acetate. After treatment with uranyl acetate the sections were stained with an alkaline solution of lead citrate (Reynolds, 1963). Specimens were examined in an AEI EM6B electron-microscope.

Determination of Predicted and Observed Vesicle Concentrations. If it is assumed that the number of vesicles *per unit length* of the long axis of a hyphal tip is constant then the concentration of vesicles in the tapered region of the tip relative to that in the region of constant (maximum) hyphal diameter may be calculated from the following equation,

$$V_P = \frac{R^2}{r^2}, \tag{1}$$

where V_P = the predicted relative vesicle concentration (vesicles per unit volume of protoplasm), R^2 is the square of the mean radius of the hypha in the region of constant diameter (25—50 μm from the tip in cot 3, Fig. 11) and r^2 is the radius of the hypha at any given point from the tip. The hyphal radius was measured on photographs of mid-longitudinal sections 0.5 μm from the tip and then at successive 1 μm intervals. The predicted relative change in vesicle concentration was calculated from these measurements using Eq. (1); V_P will of course be 1 in the region of constant hyphal diameter since $R^2 = r^2$.

The relative change in the *observed* vesicle concentration in the tip (V_o) may be calculated from the following equation,

$$V_o = \frac{V_t}{V_k}, \tag{2}$$

where V_t = vesicle concentration at any given point from the tip and V_k = the mean concentration of vesicles in the region of constant hyphal diameter. The observed variation in vesicle concentration was calculated from photographs of

the same mid-longitudinal hyphae sections used to calculate the change in the predicted vesicle concentration. Each hypha was divided along its length, into 1 μm wide segments, starting at the tip. The area of each segment was determined, the number of vesicles per segment counted and the concentration of vesicles per unit area calculated.

"*Extension Zone*" *Measurements.* The length of the apical tapered region of leading hyphae was measured on enlarged prints of photographs of glutaraldehyde fixed colonies. The photographs were taken with an Apophot (Nikon) photomicroscope.

Results

"Extension Zone" Measurements

It has been shown that the phototropic response and hence extension of stage I sporangiophores of *Phycomyces blakesleeanus* is restricted to the tapered portion of the hyphal tip (Trinci and Halford, unpublished observation). Thus it seems reasonable to assume that extension of mycelial hyphae is similarly restricted to the tapered region of the tip.

The mean length of the tapered region of leading hyphae of wild type and spco mutants of *Neurospora crassa* were determined (Table 1). There was a direct relationship (Fig. 1) between colony radial growth rate (and hence growth rate of leading hyphae) and the length of the tapered region of the hyphal tip (*i.e.* the extension zone).

From present and previous measurements (Trinci, 1973) it is possible to estimate the time required for a hypha to expand from its minimum to

Table 1. Mean colony radial growth rate, mean length of the apical tapered region of leading hyphae and mean time required for "extension zone" expansion

Strain	Mean colony radial[a] growth rate (K_r, μm/min)	Mean length of tapered[b] region of hyphal tip (extension zone) (E, μm)	"Extension zone" expansion time. Period required for a hypha to expand from its minimum to its maximum diameter (E/K_r, min)
SYR-17-3A (wild-type)	35.9	32.6 ± 11.2	0.9
spco 1	4.9	7.8 ± 1.9	1.6
spco 3	3.6	5.8 ± 1.2	1.6
spco 9	16.6	16.0 ± 2.5	1.0
spco 12	1.2	2.2	1.9
cot 3	18.5	26.5 ± 3.8	1.4

[a] From Trinci (1973).

[b] Each result is the mean of 8 to 20 measurements. Correlation coefficient, colony radial growth rate: length of tapered region of hyphal tip (K_r/E) = 0.997.

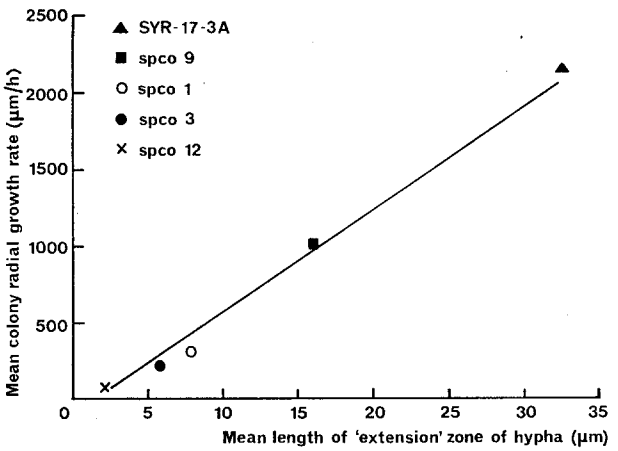


Fig. 1. Relationship between colony radial growth rate (Trinci, 1973) and the mean length of the tapered region of leading hyphae

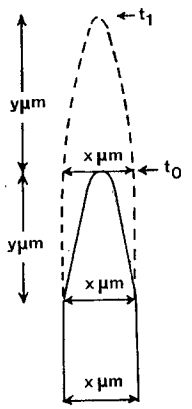


Fig. 2. Expansion in hyphal diameter: y length of the tapered region of hyphal apex (extension zone); t_0 position of hyphal tip at time, t_0; t_1 position of hyphal tip at time, t_1; x final hyphal diameter. Extension zone expansion time $= t_1 - t_0$

its maximum diameter (Fig. 2); this period will be called the extension zone expansion time. Although the strains studied grew at very different rates, their extension zone expansion times were strikingly similar (Table 1).

Hyphal Tip Ultrastructure

Figs. 3—5 show the ultrastructure of the hyphal tips of some of the strains studied. Hyphae of strain spco 9 differed from all the other strains

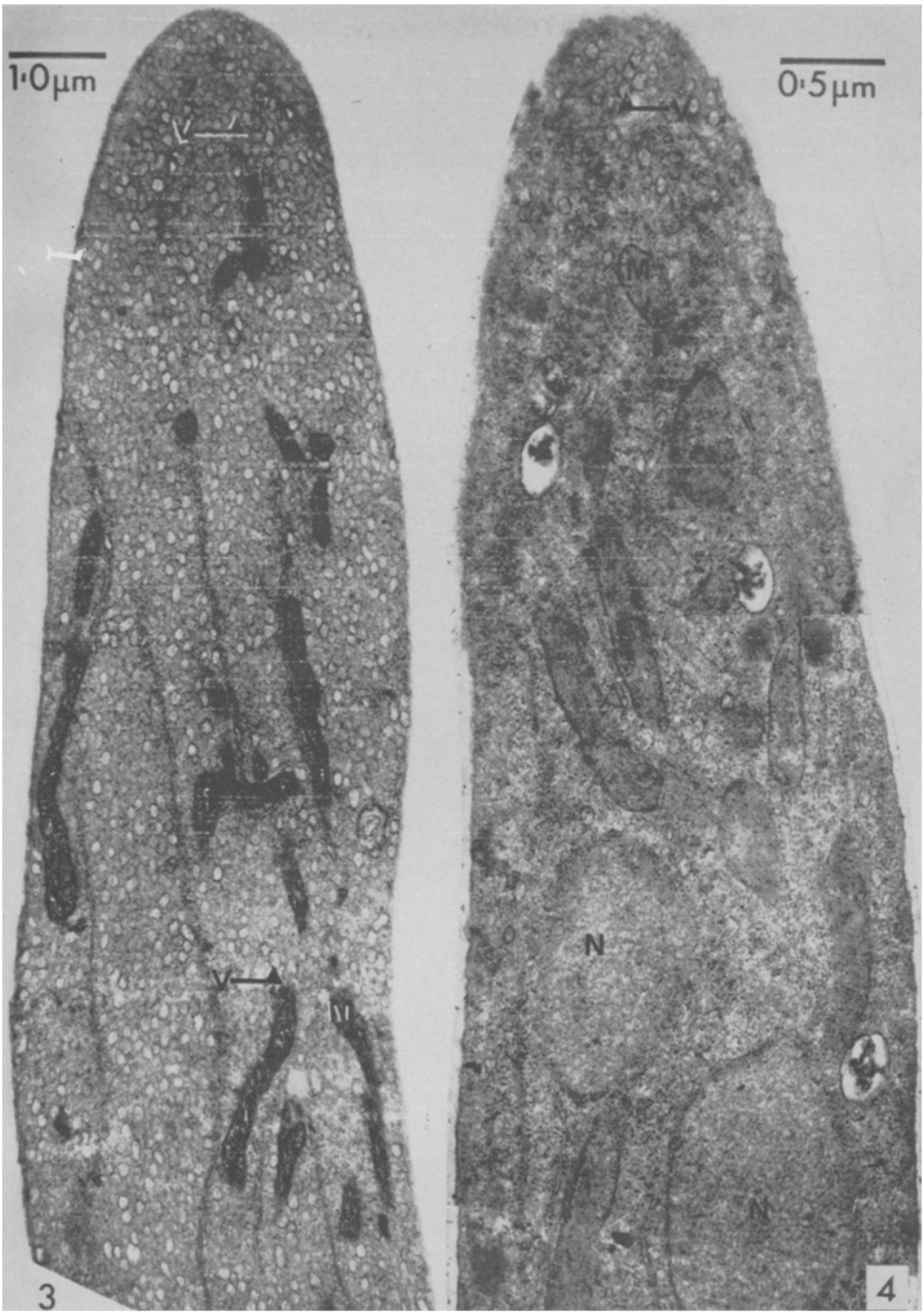


Fig. 3. Longitudinal section of a hypha of spco 9; *V* vesicles, *M* mitochondrion

Fig. 4. Longitudinal section of a hypha of spco 12; *N* nucleus

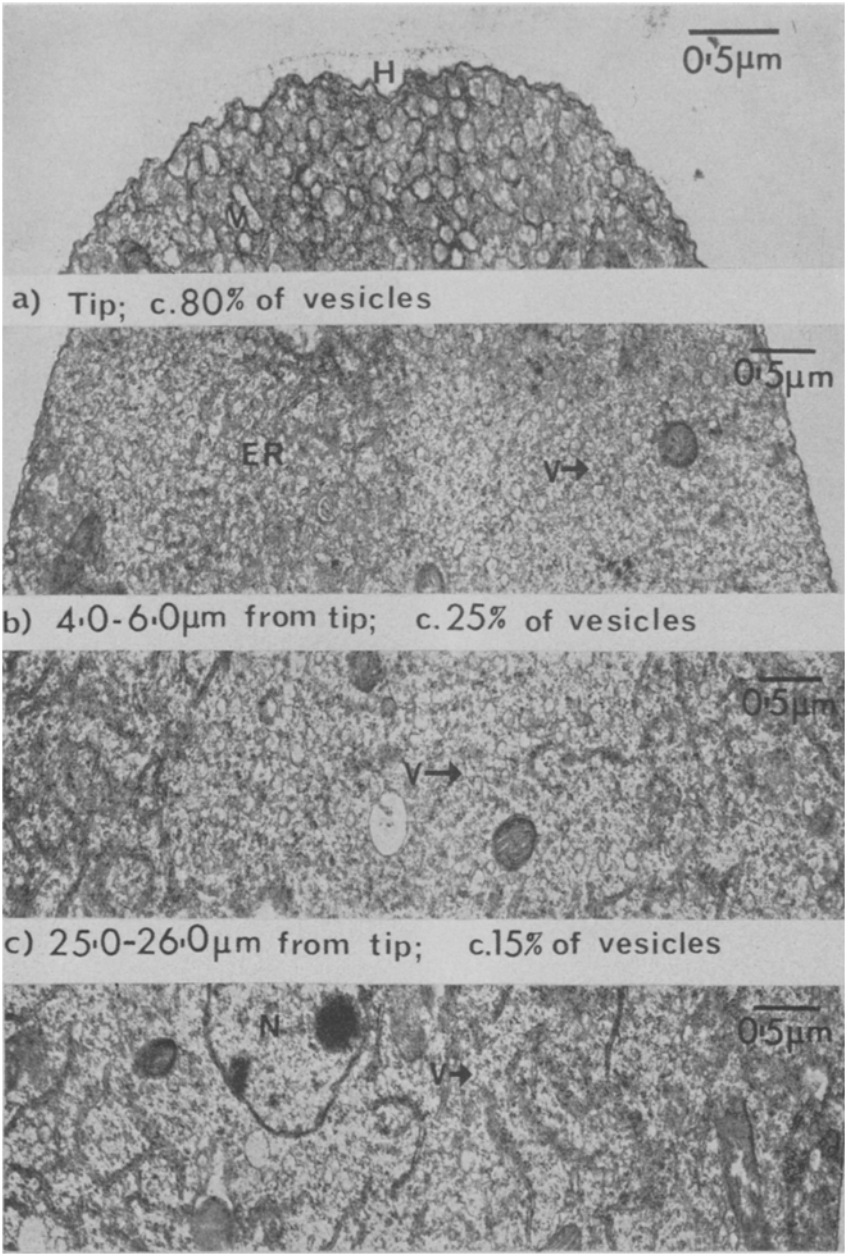


Fig. 5a—d. Selected regions of a single longitudinal section of a leading hypha of cot 3; *ER* endoplasmic reticulum; *H* tip wall; *V* vesicles; *N* nucleus. The percentage of the shown area occupied by vesicle has been calculated

Table 2. Vesicle length, vesicle concentration and organelle distribution in the tips of wild type and spco strains of *N. crassa*

Strain	Mean vesicle[a] length in terminal 10 μm of hypha (nm)	Mean maximum diameter of hyphae sectioned (μm)	Vesicle conc. in terminal 1 μm of tip (vesicles/μm³)	Estimated rate[b] at which vesicles fuse with protoplasmic membrane (vesicles/min)	Mean distance (μm) from tip[c] to first observation of:		
					Endoplasmic reticulum	Mitochondria	Nuclei
SYR-17-3A (wild type)	108 ± 24	10.3	450	37762	1.3	1.0	11.8
spco 1	143 ± 54	2.0	438	1113	4.7		10.5
spco 3	88 ± 14	1.8		1098			
spco 9	148 ± 33	3.8	450	6794	4.6	2.8	25.0
spco 12	79 ± 13	2.0	462	1278	1.6	1.3	3.3
cot 3	134 ± 26	10.4	688	8710	2.4	6.5	33.0

[a] Except for cot 3 the values are the mean of at least 100 measurements made on 3 different hyphae.

[b] Calculated from colony radial growth rates and hyphal diameters previously determined (Trinci, 1973) and present vesicle measurements.

[c] Mean value for both longitudinal and serial transverse sections.

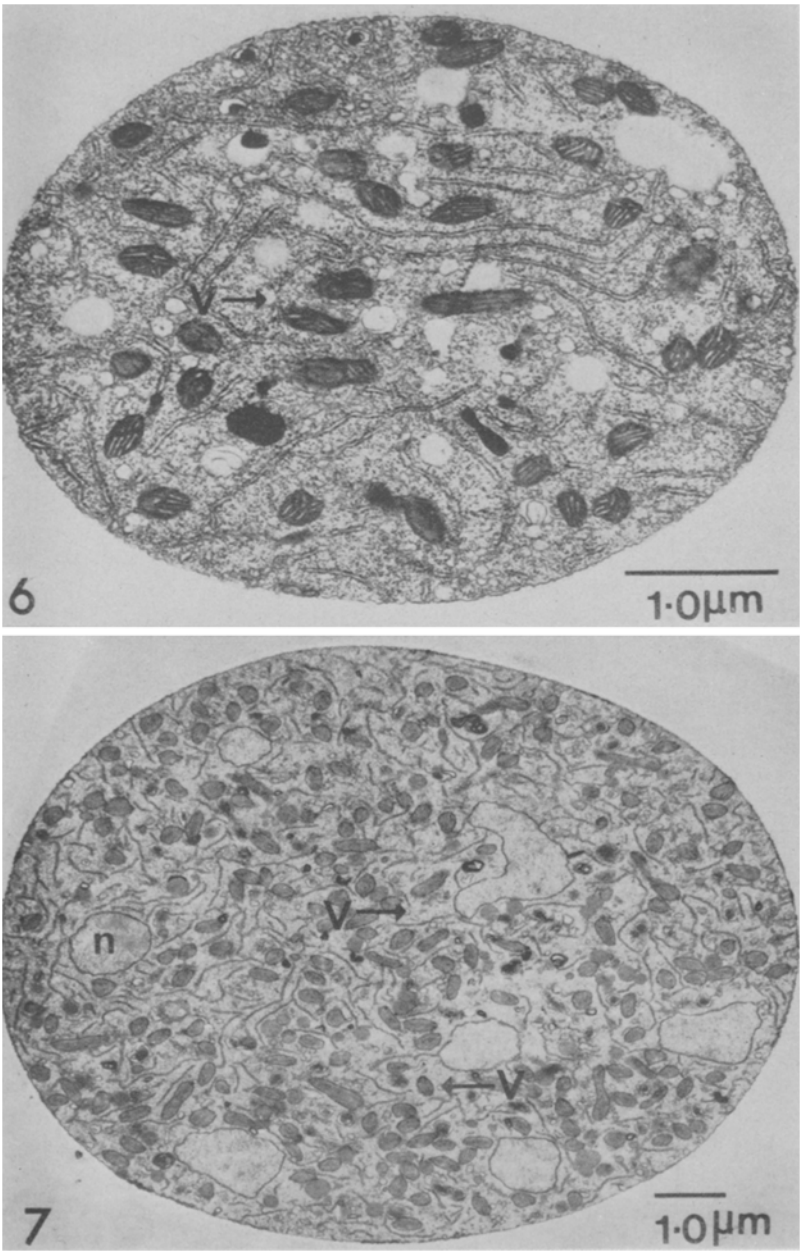


Fig. 6. Transverse section of a branch of a spco 9 hypha in apical 500 μm region

Fig. 7. Transverse section of spco 9 leading hypha about 250 μm from the tip (see Fig. 3). Note the random distribution of vesicles

in that a proportionally longer region of their tips contained a high concentration of vesicles (Figs. 3 and 13). This was the only *significant* ultrastructural difference detected between the strains. Mitochondria and endoplasmic reticulum were observed within a few μm of the tips of hyphae but nuclei, particularly in cot 3 and spco 9, were first observed at some distance from the tip (Table 2, Figs. 3—5). The concentration of endoplasmic reticulum and mitochondria in the protoplasm increased distally from the tips. The most definitive information about the distribution of organelles in a hypha is obtained from serial transverse sections of its tip; such sections showed that in spco 9 endoplasmic reticulum was situated slightly closer to the tip than mitochondria (Fig. 13). Some of the longitudinal sections of hyphae of cot 3 also showed this same sequence of organelle distribution (Fig. 11). The more distal regions of the hyphal apex appeared to be particularly well endowed with rough endoplasmic reticulum (6, 7 and 8). The endoplasmic reticulum was more or less evenly dispersed throughout the diameter of the hypha. There was an abundance of free ribosomes in the cytoplasm (Figs. 6 and 8).

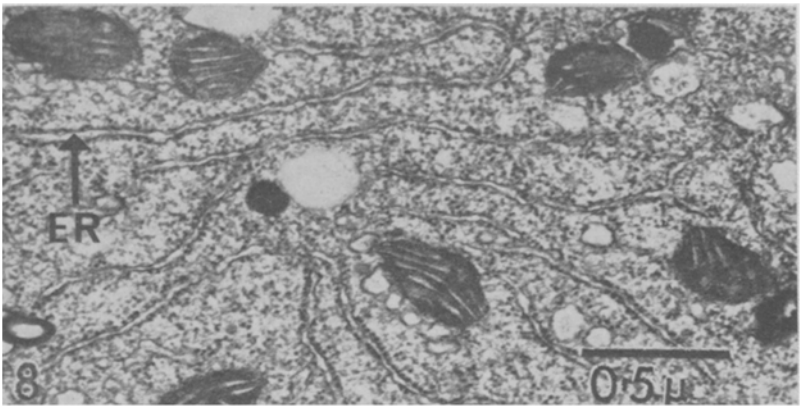
The vesicles were rarely truly spherical in shape (Figs. 5a and 9). Their mean length and concentration in the terminal 1 μm of the hyphal tip are shown in Table 2. The mean length of the vesicles in each strain did not vary over at least the terminal 10—20 μm of the hyphal tip (Fig. 13).

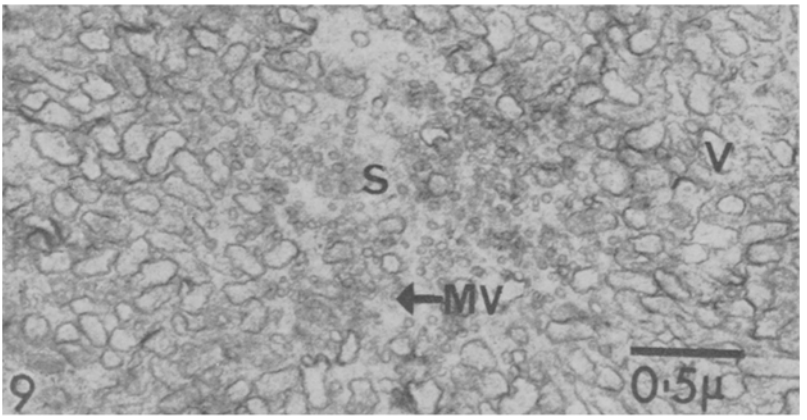
The vesicles were usually more or less uniformly distributed throughout the cross sectional area of the hypha (Figs. 5 and 7). However, a spitzenkörper (Grove and Bracker, 1970) was observed in spco 9 (Fig. 13) which was made up of microvesicles (38 ± 3 nm in diameter) and a region of low vesicle concentration (Figs. 9 and 10). The Spitzenkörper in spco 9 was spherical and had a diameter of about 2 μm.

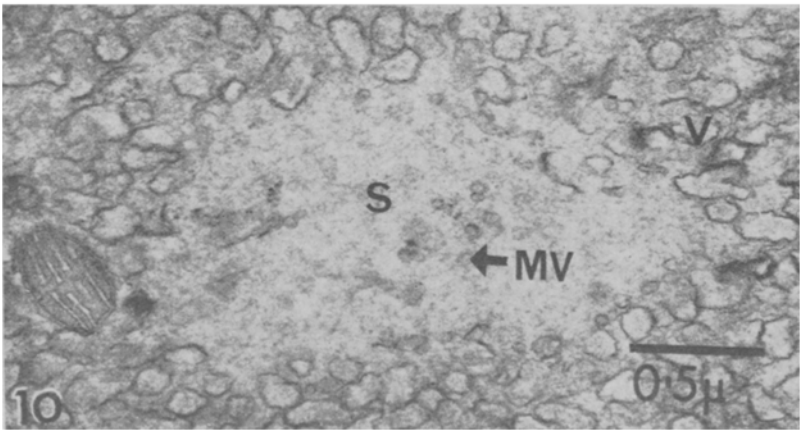
The vesicles apparently fused with the protoplasmic membrane at the tip of the hypha (Fig. 5a). If it is assumed that all the protoplasmic membrane of a hypha is derived from vesicles fusing with it at the tip, it is possible to estimate the rate of supply of vesicles necessary to maintain the observed rate of expansion of the protoplasmic membrane (Table 2). Even higher rates of vesicles supply would have to be postulated for cultures grown at 37°C since at this temperature the wild type has a colony radial growth rate in excess of 6 mm per h.

Change in Vesicle Concentration at the Tip

The percentage of the volume of a hypha of cot 3 which was occupied by vesicles was determined from a photograph of a mid-longitudinal section. Fig. 5 shows selected regions of this hypha. The plot of % of volume occupied by vesicles against distance from the tip had a very







Figs. 8—10

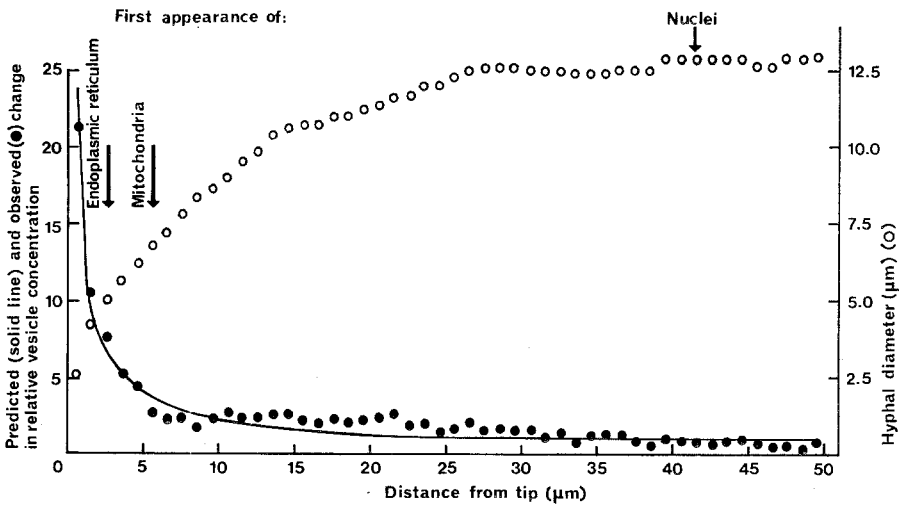


Fig. 11. Predicted (solid line) and observed (•) change in vesicle concentration in a mid-longitudinal section of a cot 3 hypha. Predicted vesicle concentration calculated using Eq. (1), observed vesicle concentration calculated using Eq. (2). Hyphal diameter (○)

similar slope to that shown in Fig. 11. About 80% of the volume of the apical 1 μm of the hypha was occupied by vesicles (Fig. 5a). This value fell sharply (see Fig. 11) to about 10%, 10 μm from the tip and 2 to 3%, 50 μm from the tip (Fig. 5d).

The rate of extension of a hypha is presumably a direct function of the rate at which vesicles fuse with the protoplasmic membrane at its tip. The distribution of vesicles in the tip of a hypha may reflect a steady state condition between the rate at which vesicles are generated and transported to the tip from the distal cytoplasm and the rate at which they fuse with the hyphal apex.

It is possible to postulate that the observed change in vesicle concentration at the hyphal apex (Figs. 3—5) results from the fact that the number of vesicles *per unit length* of the long axis of the hypha remains approximately constant as the vesicles are transported to the tip through

Fig. 8. Cytoplasm of a spco 9 hypha showing rough endoplasmic reticulum (*ER*) and free ribosomes. From apical 500 μm of hypha

Fig. 9. Spitzenkörper (*S*) in the tip of a spco 9 showing vesicles and microvesicles (*MV*). About 1625 nm from hyphal tip

Fig. 10. Spitzenkörper in the tip of a spco 9 hypha showing the central region which is almost devoid of vesicles. About 4250 nm from tip

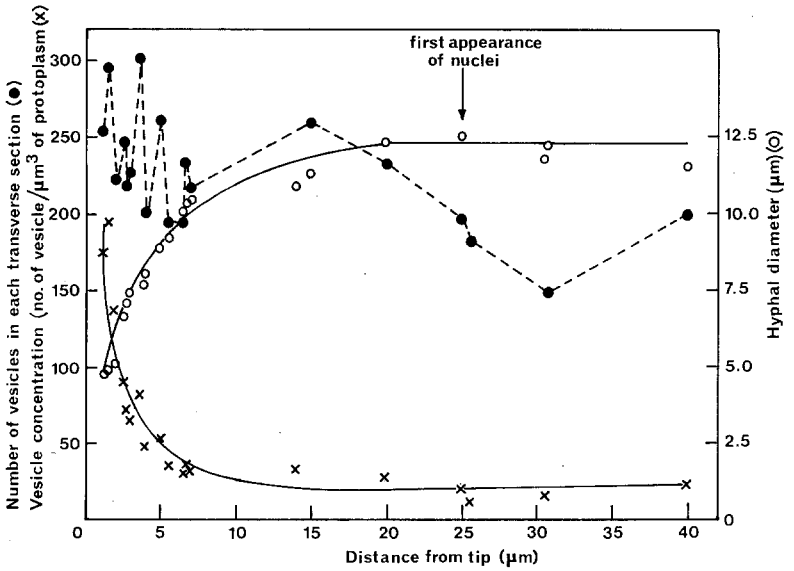


Fig. 12. Vesicle concentration (×), vesicle number (•) and diameter (○) of serial transverse sections of the tip of a leading hypha of cot 3

the tapered region of the hypha. If the observed increase in vesicle concentration at the tips of hyphae is due to this type of concentration effect it should be possible to use measurements of hyphal diameter to predict the rate of change in vesicle concentration [Eq. (1)]. Fig. 11 shows the predicted and observed change in relative vesicle concentration in the terminal region of a hypha of cot 3 (which has wild type characteristics at this temperature); the measurements of vesicle concentration and hyphal diameter were made on a mid-longitudinal section of a hypha, parts of which are shown in Fig. 5. The observed change in vesicle concentration (Fig. 11) is expressed relative to the mean vesicle concentration in the region (25—50 μm from tip) of constant hyphal diameter [Eq. (2)]. The predicted change in relative vesicle concentration was calculated using Eq. (1). Eq. (1) will only be a reliable prediction of the relative change in vesicle concentration if the following assumptions are valid:

a) the number of vesicles per unit tip *length* is constant;

b) the vesicles only fuse with the protoplasmic membrane at the extreme tip of the hypha, i.e. there is no significant reduction in vesicle number in the tapered part of the tip due to fusions with the lateral wall of the extension zone;

c) the vesicles are only transported towards the tip;

d) the groundplasm may move *towards* and *away* from the tip;

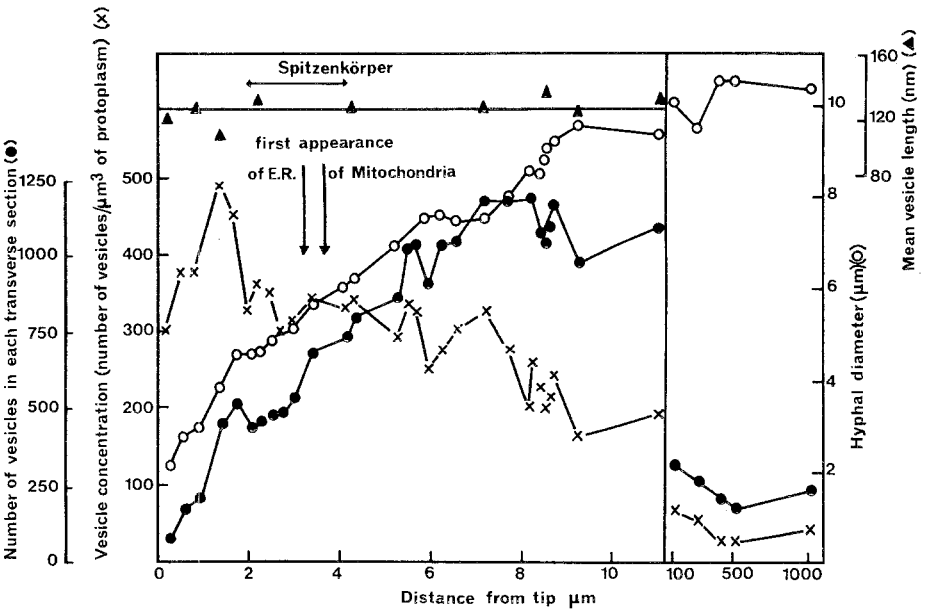


Fig. 13. Vesicle concentration (×), vesicle number (•) and diameter (○) of serial transverse sections of the tip of a leading hypha of spco 9. Vesicle length (▲), Spitzenkörper location and first appearance of endoplasmic reticulum and mitochondria

e) there is no significant generation of vesicles in the apical 50 μm of cytoplasm.

Analyses of longitudinal sections of hyphal tips of SYR-17-3A, spco 1 and 12 gave substantially the same result as for cot 3 (Fig. 11) although in these strains the fit between the observed and predicted change in vesicle concentration was not quite as good.

It should be possible to test the above hypothesis by cutting serial transverse sections of a hyphal tip and counting the number of vesicles in each section. If the hypothesis is substantially correct there should be no significant variation in the number of vesicles in each section. Fig. 12 shows the results of an analysis of serial transverse sections of a hyphal tip of cot 3; the sections were all assumed to be 80 nm thick. The number of vesicles per section was in fact more or less constant in the tapered region of this hypha. Although an attempt was made to cut and select sections of uniform thickness there would undoubtedly be some variation in section thickness. This may be reflected in the observed variation in vesicle number per section. Fig. 12 clearly shows that the increase in vesicle concentration at the hyphal tip is correlated with a decrease in hyphal diameter.

The distribution of vesicles in the tips of hyphae of spco 9 was quite different to that observed in other strains. The maximum or near maximum concentration of vesicles was first attained some 6 μm from the hyphal tip; it is difficult to imagine how any more vesicles could in fact be accommodated in the terminal 6 μm of the hypha (Figs. 3 and 13). In spco 9, unlike cot 3, the number of vesicles per transverse section was clearly not constant over the terminal 10 μm of the hypha (Fig. 13). Spco 9 also differed from cot 3 in that the concentration of vesicles in the cytoplasma decreased progressively in a distal direction even in the region where the hyphal diameter was constant (Fig. 13). A concentration of about 25 vesicles per μm^3 was first attained some 25 μm from the tip of the cot 3 hypha (Fig. 12) but not until 300 μm from the tip of the spco 9 hypha (Fig. 13).

Discussion

Studies of wild type and mutant strains of *Neurosporsa crassa* (Trinci, 1973; present results) have shown that the rate of extension of a hypha is directly related to each of the following parameters: length of the peripheral growth zone, hyphal diameter, length of the apical compartment, length of the intercalary compartment and length of the extension zone. The length of the peripheral growth zone (in the absence of a change in specific growth rate, Trinci, 1973) is almost certainly the crucial parameter which determines extension rate since it probably regulates the supply of vesicles to the tip. The changes in the other parameters are probably secondary events which result from changes in the length of the peripheral growth zone.

Vesicles may concentrate in the extension zone of a hypha because they are mainly generated by the cytoplasm in this region. However, we consider this hypothesis unlikely since it implies very fast rates of synthesis of membrane and wall precursors. About 38000 vesicles per minute are required to maintain the extension of wild type hyphae at 25°C. The present results indicate that the observed increase in vesicle concentration in hyphal tips may largely result from the transport of vesicles at a constant rate into the tapered region of the tip where the volume of groundplasm per unit hyphal length is decreasing at a progressive rate. The suggestion that there is a movement of groundplasm towards and away from the tip is supported by direct observations of cytoplasmic streaming in hyphae (Burnett, 1968). A bidirectional movement of groundplasm would imply that the rate of cytoplasmic streaming towards the tip exceeds the rate of hyphal extension; this is generally observed to be the case (Burnett, 1968). The undirectional nature of vesicle transport is presumably a consequence of their ultimate fusion with the protoplasmic membrane at the tip. Bartnicki-Garcia (1973) has re-

cently suggested the vesicles may be transported by electrophoretic movement.

Fig. 11 is consistent with the hypothesis that most of the vesicles observed in the tips of cot 3 hyphae are generated in cytoplasm distal to the terminal 50 μm. Trinci and Collinge (1973) showed lines of vesicles traversing the septal pores of spco 1 hyphae; the vesicles had a similar diameter to those observed in the hyphal tips. These observations suggest that vesicles are generated throughout the cytoplasm of the peripheral growth zone (Trinci, 1973) and transported apically. However, the slight increase in vesicle concentration which was observed in the region of the cot 3 hypha which had a constant diameter suggests that vesicles are also generated in the apical 50 μm of this hypha (Fig. 11).

The assumption that vesicles do not fuse with the lateral walls of the extension zone is not consistent with the observed increase in hyphal diameter in this region (Fig. 2). However, most of the vesicles may in fact fuse with the extreme tip of the extension zone.

The usual shape of the curve showing the change in vesicle concentration in the hyphal tip (Figs. 11 and 12) almost exactly parallels the rate of incorporation of N-[^{3}H] acetyl D-glucosamine into the tips of *Neurospora crassa* hyphae (Gooday, 1971). The vesicles may thus be intimately involved in "the maintenance of a steep descending gradient in relative rate of area expansion within the apical dome (which) appears to be the essential basis for the generation of cylindrical form in tip-growing cells" (Green, 1969).

The results obtained are also consistent with the hypothesis that vesicles do not normally fuse with the protoplasmic membrane situated below the extension zone. That such fusions do not usually occur is suggested by the observation that lomasomes are not an extensive feature of well fixed hyphae (Grove and Bracker, 1970); lomasomes or some similar membranous proliferation would presumably result from any substantial number of fusions of vesicles with the protoplasmic membrane below the extension zone. It is possible the fusions predominantly occur in the tapered extension zone of the hyphal tip because this is where the vesicles are most likely to come in physical contact with the protoplasmic membrane.

All the results to date suggest that vesicles are primarily concerned with wall formation (primary wall formation?) which is linked to membrane and wall extension, *e.g.* tip growth, budding or branch insitation. The observed sub-apical increase in thickness (Hunsley, 1973) associated with maturation of the wall (secondary wall formation?) may not involve vesicles. Thus, it is possible that precursors are transported to the growing wall by both membranous (vesicles) and non-membranous systems and that the former is primarily concerned with hyphal extension.

The results obtained with spco 9 (Fig. 13) suggest that vesicles accumulate at its tip either because they fuse with the protoplasmic membrane at a slower rate than in the wild type or that a high proportion of vesicles is generated in the region 10 to 400 μm from the tip. The spco 9 strain may be particularly useful in studies of vesicle isolation.

The relative constancy of the extension zone expansion time (Table 1) in strains which grow at widely different rates may reflect some inherent property of the vesicle which has not been altered by the mutations.

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