An Autoradiographic Study of Hyphal Growth of Some Fungi

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(Accepted for publication 7 May 1971)

SUMMARY

Incorporation of radioactive glucose and N-acetyl glucosamine into the walls of growing fungal hyphae has been studied using light microscopic autoradiography. Autoradiographs of Phytophthora parasitica with glucose as substrate and Neurospora crassa and Schizophyllum commune with glucose or N-acetyl glucosamine as substrate show that most of the incorporation after incubation times as short as 1 min. is at the extreme hyphal tip. N-Acetyl glucosamine is incorporated less into the subapical region than glucose, and N. crassa shows less subapical incorporation of both substrates than the other two fungi.

INTRODUCTION

It is generally accepted that a fungal hypha grows by deposition of material at its tip (Burnett, 1968) although the details of this process are poorly understood. Recently Bartnicki-Garcia & Lippman (1969) demonstrated that the inclusion of N-acetyl glucosamine (as polysaccharide) was greatest at the germ-tube tip of spores of Mucor rouxii. An autoradiographic study was made of the incorporation of glucose into the hyphal walls of Phytophthora parasitica and of both glucose and N-acetyl glucosamine into the hyphal walls of Schizophyllum commune and Neurospora crassa. Both the chemical nature (Bartnicki-Garcia, 1966; Mahadevan & Tatum, 1967; Niederpruem & Wessels, 1969) and the molecular architecture (Hunsley & Burnett, 1968, 1970) of the walls of these fungi have been investigated.

METHODS

Media and culture methods. The cultures of Phytophthora parasitica Dastur, Neurospora crassa Shear & Dodge and Schizophyllum commune Fries were those used in previous studies (Hunsley & Burnett, 1968, 1970). The fungi were grown at 20° on the glucose + asparagine medium described for P. parasitica by Hunsley & Burnett (1970) but with the addition of biotin (5 ng./l.) for N. crassa and adjusted to pH 6.7 for S. commune. Several techniques were tried for preparing material for autoradiography so that the mycelium retained its spatial orientation during handling. The most satisfactory method proved to be to grow the fungus on a 25 mm. diameter filter disc (Millipore GS, 0.22 μm. pores) that had been infiltrated with agarose (Seravac Ltd) by immersion in a hot 1% (w/v) solution and drained on to filter paper. The filter disc was laid on the appropriate medium solidified with agar (Oxoid no. 3, 2%, w/v) and inoculated in the centre with a small piece of mycelium. The presence of the agarose made the filter disc sufficiently adhesive to hold the mycelium of P. parasitica and S. commune in place during handling, but the N. crassa mycelium sometimes floated free after fixation and had to be mounted separately.

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Vol. 67. No. 1, was issued 15 September 1971
Methods used to label the hyphae with radioactive D-glucose or acetyl D-glucosamine. 

\([3-3^\text{H}]\text{D-glucose } (1.1 \text{ Ci/mm}), [4-3^\text{H}]\text{D-glucose } (7.2 \text{ Ci/mm}), [5-3^\text{H}]\text{D-glucose } (0.9 \text{ Ci/mm}), [6-3^\text{H}]\text{D-glucose } (2 \text{ Ci/mm}) \text{ and } N-[3^\text{H}]\text{acetyl D-glucosamine } (243 \text{ mCi/mm}) \text{ were obtained from the Radiochemical Centre, Amersham, Buckinghamshire. When } N\text{-acetyl glucosamine was the substrate the filter discs with adhering fungus were transferred from the agar medium to float on liquid medium of the same constitution, to which had been added } 50 \mu\text{Ci of the radioactive substrate. However, when glucose was the substrate, and in the earlier experiments with } N\text{-acetyl glucosamine, the radioactive carbohydrate was added to a glucose-free medium to prevent dilution of the isotope, and so the filter discs were floated on a series of media with the glucose progressively being replaced by isotonic potassium chloride over } 20 \text{ min. before finally being floated on the radioactive medium. Preliminary experiments used incubation times of } 10 \text{ min., with or without a 'cold chase' of non-radioactive substrate for a further } 10 \text{ min. which was calculated to dilute the radioactive compound by a factor of } 1 \times 10^4 \text{ and was achieved by transferring the filter discs to the appropriate isotonic medium. In later experiments these times were cut down to } 1 \text{ min. with or without a } 1 \text{ min. 'cold chase'. At the end of the incubation time the mycelium was fixed by immersion in ice-cold ethanol for } 5 \text{ min., followed by extraction in } 60\% \text{ (v/v) ethanol for } 1 \text{ h. at } 4^\circ. \text{ The filter discs were then cut into four segments and the cytoplasm dissolved out by heating in ethanolic sodium hydroxide } (5 \text{ min., } 100^\circ, \text{ in } 1 \text{ M-NaOH + ethanol, } 1:2) \text{ as described by Bartnicki-Garcia & Lippman (1969). Phase contrast microscopy confirmed that only the hyphal walls remained. The pieces of filter disc were left in acetone overnight to remove nearly all of the filter matrix, leaving thin transparent membranes with the adhering mycelial ghosts. These were soaked in water and mounted on subbed microscope slides.}

**Autoradiographic techniques.** Stripping film autoradiography (applying Kodak AR-10 as recommended by the manufacturers) was used as it was found to be more satisfactory for the material than techniques using liquid emulsion. Several replicate preparations were made for each treatment, and these were developed after varying exposure times between 2 days and 20 days so as to obtain autoradiographs that were suitable for grain counting. The autoradiographs were developed for 4 min. (Kodak D-19) at 20°, rinsed and fixed until clear (Ilford Hypam fixer). The addition of an acid hardener (Ilford Hypam hardener) to the fixer was necessary to prevent the loosening of the film that otherwise occurred with the local soft water. As recommended by Rogers (1967) each batch of autoradiographs was accompanied by a control of non-radioactive mycelium and by a control in which the emulsion had been exposed to light. After processing, the autoradiographs were washed, dried and stored dry. For examination a drop of water was added to the centre and covered by a cover-slip which was afterwards removed and the slide redried.

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**Fig. 1.** Autoradiographs of apical regions of hyphal walls

*a)* *Phytophthora parasitica* incubated for 1 min. in \([5-3^\text{H}]\text{D-glucose.}*

*b)* *Schizophyllum commune* incubated for 10 min. in \([5-3^\text{H}]\text{D-glucose.}*

*c)* *S. commune* incubated for 10 min. in \(N-[3^\text{H}]\text{acetyl D-glucosamine.}*

*d)* *Neurospora crassa* incubated for 1 min. in \([5-3^\text{H}]\text{D-glucose.}*

*e)* *N. crassa* incubated for 1 min. in \(N-[3^\text{H}]\text{acetyl D-glucosamine.}*

*f)* *P. parasitica* incubated for 10 min. in \([5-3^\text{H}]\text{D-glucose followed by 10 min. in a 'cold chase' of unlabelled glucose.}*

*g)* *S. commune* incubated for 10 min. in \([5-3^\text{H}]\text{D-glucose followed by 10 min. in a 'cold chase' of unlabelled glucose.}*

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Examination of the autoradiographs and quantitative estimation of radioactivity. The autoradiographs were photographed either by transmission or phase contrast illumination, and were expressed quantitatively by counting the silver grains over selected areas of the photographs and then obtaining the specific activities of grains/unit area by cutting and weighing the paper. The silver grains over the apical 75 μm of each hypha were counted, ignoring those hyphae that had branches, septa or any other hyphae close by. Photographs of hyphal tips were also cut out, mounted in a carrier and scanned with a Joyce Loebel Chromoscan densitometer in its reflectance mode with integration facilities, using slits and point sources of light. Although this technique produced a graphical representation of grain densities of individual tips, it was less convenient than counting silver grains for comparing a number of tips from different treatments. Several series of autoradiographs were made of the same mycelial wall preparations of all three fungi before and after they had been incubated with hydrolytic enzymes. Preliminary experiments were done by stripping the emulsion film from the slide after the first autoradiograph, then incubating with a drop of the appropriate enzyme (as used by Hunsley & Burnett, 1970) and finally recoating with fresh emulsion. In later experiments the fungal material was protected from the photographic chemicals by coating it with a formvar film (Stubblefield, 1965) which was then removed with chloroform after the first autoradiograph, and the enzyme incubation performed as before.

RESULTS

With both glucose and N-acetyl glucosamine, incorporation of radioactive substrate was apical (Fig. 1). Fig. 2 to 6 present the results of counts of the silver grains per unit area over the hyphal tips of the three fungi. In order to compare the degrees of apical incorporation

![Graph](Phytophthora_parasitica_Glucose)

**Fig. 2.** Counts of silver grains from autoradiographs of *Phytophthora parasitica* incubated for 1 min. in [5-3H]β-glucose. ◦, Silver grains/10 μm² ± standard deviation (S.D.) (10 tips counted). ●, Silver grain count relative to that 50 to 75 μm behind the apex.
of the two substrates by the different fungi, the results are also plotted as specific activities relative to those at 50 to 75 μm. behind the apex. In each case the highest concentration of incorporated labelled substrate was over the apical 1 μm. and fell off sharply after the first 5 μm. The growth rates of the three fungi until the time of the experiment had been: *Phytophthora parasitica*, 1.2 μm./min.; *Neurospora crassa*, 8.5 μm./min.; and *Schizophyllum commune*, 3.8 μm./min. However, no attempt was made to measure the growth rate during the subsequent handling procedures.

![Graph](image)

**Fig. 3.** Counts of silver grains from autoradiographs of *Schizophyllum commune* incubated for 10 min. in [5-3H]-glucose. Symbols as for Fig. 2.

The results also show that when glucose was the substrate for *Phytophthora parasitica* and *Schizophyllum commune* there was still appreciable incorporation between 5 μm. and 75 μm. behind the tips. This contrasts with *S. commune* when N-acetyl glucosamine was substrate, and with *Neurospora crassa* for both carbohydrates as substrates. Here a much greater proportion of the incorporation was over the extreme hyphal tips.

No differences in distribution of incorporated material were observed between hyphae incubated in glucose tritiated in the 3, 4, 5 or 6 positions. However, as the tritium at the C-5 position might be expected to be the most stable during glucan synthesis, most of the work used this compound. In early preparations N-acetyl glucosamine as substrate was added to mycelium that had been washed free from glucose to lessen the chance of any dilution of the isotope by formation of unlabelled amino sugar derivatives during the experiment. Later work showed that the presence of glucose made no difference to the incorporation of the N-acetyl glucosamine, and so the experiments were then done with glucose present.

That the labelling was incorporated into insoluble polymers and not adsorbed on the walls was readily shown by ‘pulse-chase’ experiments. Here the radioactivity was diluted
out after a suitable time by transferring the mycelium to an isotonic medium containing unlabelled substrate at a 10,000-fold higher concentration than the radioactive material and then incubating for a further period of time. Fig. 1f and g show typical hyphal tips after such treatment in glucose with the highest density of incorporation being subapical after further hyphal growth. This subapical appearance was not observed in Neurospora crassa and Schizophyllum commune when a high concentration of N-acetyl glucosamine was used.

Fig. 4. Counts of silver grains from autoradiographs of Schizophyllum commune incubated for 10 min. in N-[3H]acetyl D-glucosamine. Symbols as for Fig. 2.

Fig. 5. Counts of silver grains from autoradiographs of Neurospora crassa incubated for 1 min. in [5-3H]D-glucose. Symbols as for Fig. 2.
as a 'cold chase', and the labelling remained apical. Although this carbohydrate can act as sole carbon source for these fungi, presumably the experiments did not allow them time to adapt to it.

Particularly with *Neurospora crassa*, and to some extent with the other fungi, different hyphae showed a range of intensity of labelling. It was not uncommon to find what were apparently normal hyphal tips of *N. crassa* with very little or even no labelling.

The apices of side branches of all three fungi showed localized heavy labelling like that of the leading hyphal tips. Radioactivity was also associated with developing cross-walls in the three fungi and this has been investigated for *Phytophthora parasitica* by Gooday & Hunsley (1971).

![Graph showing autoradiography results](image)

**Fig. 6.** Counts of silver grains from autoradiographs of *Neurospora crassa* incubated for 1 min. in N-[3H]acetyl D-glucosamine. Symbols as for Fig. 2.

It has not proved possible to change the spatial distribution or intensities of incorporated material in autoradiographs by extracting the mycelial wall preparations with hydrolytic enzymes. It has therefore not been possible to compare the autoradiographic labelling more directly with the 'enzymic dissection' results of Hunsley & Burnett (1970). Possibly the procedure used to obtain the wall ghosts alters the molecular configuration of the wall polymers so as to protect them from the enzymes.

**DISCUSSION**

*Interpretation of the autoradiographs*

The AR 10 stripping film has a grain size of 0.2 to 0.3 µm. and an emulsion thickness of about 3.6 µm. during exposure (Rogers, 1967). From the silver grain counts in Fig. 2 to 6 and by examining the Fig. 1 photographs for the fall-off of grain density outside the hyphae the resolution obtained appears to be close to the theoretical maximum.

Each substrate was incorporated into wall polysaccharides after a 1 min. incubation time, and the different autoradiographic patterns show that this incorporation is specific. The N-acetyl glucosamine was tritiated in the acetyl group so the molecule is apparently incorporated without hydrolysis.

In *Schizophyllum commune* and *Neurospora crassa* some of the incorporated glucose
probably represents chitin formation, but as the ratios of glucan:chitin in the cell walls are of the order of 28:1 (Niederpruem & Wessels, 1969) and 9:1 (de Terra & Tatum, 1963) respectively, this might be expected to be a minor contribution to the labelling pattern. The extraction procedure used to prepare cell-wall ghosts probably removes some of the lipids and proteins of the walls. However, the present study using glucose and N-acetyl glucosamine as substrates is concerned with the polysaccharides of the walls, which would be expected to resist this procedure.

**Apical growth**

The results from Fig. 2 to 6 clearly confirm the idea that a fungal hypha grows by localized apical wall formation and they complement the results of Bartnicki-Garcia & Lippman (1969) in showing that the highest rate of wall synthesis is in the apical 1 μm. of the hypha. Recent electron microscopy of the extreme hyphal tips of fungi (McClure, Park & Robinson, 1968; Girbardt, 1969; Grove & Bracker, 1970; Grove, Bracker & Morré, 1970) has shown that this region is characterized by an accumulation of cytoplasmic vesicles which these authors implicate in wall formation. There is a correlation between the distribution of silver grain densities seen in the present work and the distribution of vesicles seen in their photographs. However, it must be emphasized that the fungal wall is both chemically and anatomically a complex multi-component system (Hunsley & Burnett, 1970) and it is possible that different compounds will prove to have different cellular sites of synthesis, as is becoming clear in studies of biosynthesis of higher plant cell walls (Northcote, 1969).

Hunsley & Burnett (1970) have clearly shown that the extreme apices of the hyphae of all three fungi contain randomly orientated microfibrils in their walls. Since these microfibrils can be ascribed chiefly to chitin in *Neurospora crassa* and *Schizophyllum commune* and to cellulose in *Phytophthora parasitica*, the incorporated material in the extreme apices of *N. crassa* and *S. commune* with N-acetyl glucosamine as substrate and some of that in the apex of *P. parasitica* with glucose as substrate may represent microfibril formation. Some of the subapical labelling observed in these cases could represent the process of intussusception suggested by Hunsley & Burnett (1968), who showed that the dimensions of the microfibrillar elements in the walls of these three fungi, whether of cellulose or chitin, increased away from the tip.

The subapical incorporation of glucose showed that in this region there is still a considerable rate of synthesis of wall material in *Phytophthora parasitica*, less in *Schizophyllum commune* and very little in *Neurospora crassa* compared with the rate of synthesis at the extreme apices. However, Hunsley & Burnett (1970) have shown that, particularly in *N. crassa*, considerable amounts of non-fibrillar material are added to the wall in the subapical region. These authors suggest that some of this is glycoprotein in nature. This would not be expected to show much radioactive incorporation from the carbohydrates used here in the short incubation periods. In addition, it must be emphasized that the autoradiographic patterns obtained represent the rate of synthesis of wall components and cannot be expected to relate directly to the relative amounts of the components of the mature cell walls, which must also be governed by the duration of the synthetic processes.

The localization of wall synthesis in *Neurospora crassa* as shown by autoradiography may be compared with the detailed cytochemical investigation of this fungus by Zalokar (1959). It is clear that the extreme apical localization of wall synthesis shown here is not mirrored by any of his conclusions concerning the distribution of a range of enzymes and macromolecules.

The variation observed in the intensity of incorporation between different hyphal tips
Autoradiography of hyphal growth

of the same culture could reflect the periodic slowing of the growth rate of some leading hyphae and the concomitant acceleration of growth of some hyphae behind the apex, a phenomenon which is implicit in many observations of hyphal growth (e.g. Smith, 1924) but which still demands careful study and analysis.

The author thanks Professor J. H. Burnett for help and encouragement during the course of the work and in the preparation of the manuscript, and the Agricultural Research Council for financial support (A.R.C. grant AG 43/54).

REFERENCES


