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LIGHT AND ELECTRON MICROSCOPIC AUTORADIOGRAPHY OF CELL-WALL REGENERATION BY SCHIZOPHYLLUM COMMUNE PROTOPLASTS

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SUMMARY

Regenerating protoplasts of the basidiomycete *Schizophyllum commune* were labelled with radioactive N-acetylglucosamine. The label was mainly incorporated in the alkali-insoluble wall fractions R-glucan (containing some hexosamine) and chitin. Light microscopic autoradiography showed a uniformly dispersed incorporation of label in primary cells and chain-cells but emerged hyphae revealed a highly localized incorporation at the hyphal apex. Electron microscopic autoradiography showed that the label mainly appeared just outside the plasmalemma in the wall suggesting that chitin and R-glucan are synthesized at the plasmalemma/cell wall interface.

1. INTRODUCTION

A novel approach to the problem of cell-wall synthesis and morphogenesis in fungi is the study of wall regeneration by isolated protoplasts. In filamentous fungi hyphae are formed either directly from the regenerated protoplasts (primary cells) or at the end of chain-cells, which arise by repeated budding of primary cells (STRUNK 1970; VILLANUEVA & GARCÍA-ACHA 1971; PEBERDY 1972; WESSELS et al. 1976).

A chemical analysis of cell-wall regeneration by protoplasts of the basidiomycete *Schizophyllum commune* has shown that the regenerated walls contain the same wall polymers as the parental hyphal walls, viz. alkali-soluble S-glucan (α-1,3-glucan), alkali-insoluble R-glucan (β-1,3-β-1,6-glucan) and chitin (β-1,4-poly-N-acetylglucosamine) (DE VRIES & WESSELS 1975). A recent ultrastructural study, however, demonstrated a difference in wall architecture between primary cells and chain-cells on the one hand and hyphae of reverted protoplasts on the other hand (VAN DER VALK & WESSELS 1976).

To relate these differences in cell-wall construction to possible differences in cell-wall biosynthesis a study was made of the incorporation of labelled N-acetylglucosamine using autoradiography in combination with cell fractionation.

2. MATERIALS AND METHODS

2.1. Preparation and regeneration of protoplasts

Floating protoplasts of the monokaryotic strain 699 of *Schizophyllum commune* Fr. (collection Professor J. R. Raper, Harvard University, Cambridge, Mass., U.S.A.) were isolated and prepared for regeneration studies as described by DE VRIES & WESSELS (1975).

2.2. Cell fractionation

After 9 h of regeneration, N-acetyl-(D-glucosamine-1-^{14}C) (specific activity: 59 mCi/mM; Radiochemical Centre, Amersham, England) was added to the culture to give a final activity of 1.2 μCi/ml. After labelling for 30 min the cells were collected on a membrane filter (pore size 0.8 μm) and washed on the filter with a solution containing 0.5 M $MgSO_4$, 0.05 sodium maleate and 0.44 mM N-acetyl-D-glucosamine (pH 5.8). The material was then suspended in 0.05 M KH_2PO_4-NaOH buffer (pH 7.0) and clean cell walls were prepared according to the procedure of WESSELS & MARCHANT (1974). This included breakage of the cells with an X-press and washing of the walls on the centrifuge. The cytoplasmic fraction, comprising the combined supernatants, was lyophilized and analysed. The S-glucan, R-glucan and chitin fractions of the walls were prepared according to the procedure of DE VRIES & WESSELS (1975). The S-glucan fraction was hydrolysed in 1 N HCl (100°C; 6 h) and the R-glucan and chitin fractions were hydrolysed in 6 N HCl (100°C; 6 h). Hydrolysis was performed in sealed tubes. The hydrolysis products were dried (vacuum; KOH) and dissolved in water.

Total radioactivity was determined by counting samples in a scintillation solution containing 100 g naphtalene and 7 g 2,5-diphenyloxazole (PPO) per litre of dioxane. Thin-layer chromatography of the hydrolysates was done on Cellusose F coated plates (Merck) with pyridine-ethylacetate-acetic acid-water (5:5:1:3, v/v; solvent A) and with methanol-pyridine-acetic acid-water (6:6:1:4, v/v; solvent B). Radioactivity on thin-layer chromatograms was measured with a Berthold thin-layer scanner.

2.3. Autoradiography

After 3 and 9 h of regeneration N-(acetyl-^{3}H)-D-glucosamine (specific activity: 680 mCi/mM; Radiochemical Centre, Amersham, England) was added to the culture to give a final activity of 30 μCi/ml. After labelling for 30 min the material was fixed in a solution containing 2% (w/v) glutaraldehyde, 0.1 M sodium cacodylate, 0.2 M $MgSO_4$ and 0.44 mM N-acetyl-D-glucosamine (pH 5.8; 0.68 Osm) for 2 h at room temperature.

For light microscopic autoradiography the glutaraldehyde-fixed material was washed four times on the centrifuge (10 min; 3000 g) with 0.44 mM N-acetyl-D-glucosamine. A suspension of the washed cells was brought on gelatin-chrome alum coated slides and, after drying, the slides were coated with Kodak AR-10 stripping film (ROGERS 1967). After exposure for 6 days (4°C)

the autoradiographs were developed in Kodak D-19 developer for 5 min at 18 °C, rinsed in water and fixed in Kodak Unifix. Finally they were stained for 1 min with a 1:45 (v/v) mixture of 1 % toluidine blue and 1 % borax, rinsed in water, dried in air, and mounted in Entellan (Merck).

For electron microscopic autoradiography the glutaraldehyde-fixed cells (9 h of regeneration) were washed three times on the centrifuge (3000 g; 10 min) in a solution containing 0.1 M sodium cacodylate, 0.35 M $MgSO_4$ and 0.44 mM N-acetyl-D-glucosamine (pH 7.2). The material was postfixed in a solution containing 1 % (w/v) OsO_4, 0.35 M $MgSO_4$ and 0.1 M sodium cacodylate (pH 7.2). After dehydration in a graded series of ethanol solutions the material was embedded in Epon 812. Sections showing grey interference colors (thickness about 50 nm) were mounted on formvar/carbon coated grids, stained with uranyl acetate and lead citrate (REYNOLDS 1963), covered with a thin layer of carbon and mounted on clean slides with double sided adhesive tape (Scotch). The sections were then covered with a monolayer of Ilford L4 Nuclear Research emulsion applying the wire-loop technique (CARO & VAN TUBERGEN 1962). Autoradiographs were stored at 4 °C for 2–5 months in light-tight boxes containing silica-gel and developed in Kodak D-19 developer (3 min; 19 °C) of for fine-grain development according to the procedure of WISSE & TATES (1968), except for a reduction of the developing time to 5 min. The latter method involves gold latensification and elon-ascorbic acid (GEA) development. Specimens were then fixed in 24 % sodium thiosulphate (2 min; 20 °C), rinsed in water, and dried at room temperature.

GEA-developed autoradiographs were used for quantitative analysis. The position of the silver grains over and adjacent to the cell wall was determined by recording the distance of the centre of the grain to the plasmalemma. Grains were counted within a "cut-off" distance of 0.8 μm from the plasmalemma. Only micrographs, showing cells with diameters exceeding 2.2 μm, were used for measurements. In addition the thickness of the total wall and its compact inner layer were measured at the position of each grain.

3. RESULTS

3.1. Cell fractionation

During growth of regenerating protoplasts on N-acetyl-(glucosamine-1-^{14}C) for 30 min, 2 per cent of the label was incorporated and most of it appeared in the alkali-insoluble wall fractions (*table 1*). To determine which of the wall monomers was radioactive, the R-glucan and chitin fractions were hydrolysed with 6 N HCl and chromatographed in solvent A. Glucosamine was the only labelled substance present.

The labelled material of the cytoplasmic fraction was completely soluble in 60 % (v/v) ethanol. Thin-layer chromatography, using solvent B, showed that nearly all of the radioactivity was present in one distinct spot, which showed UV-fluorescence and had a R_f value identical to uridine-diphosphate-N-acetylglucosamine.

Table 1. Distribution of label from N-acetyl-(D-glucosamine-l-^{14}C) in cell fractions of regenerating protoplasts of *Schizophyllum commune*.

Cell fraction	Incorporation (%)*
Cytoplasm	14.7
S-glucan	0.4
R-glucan	30.9
Chitin	53.6

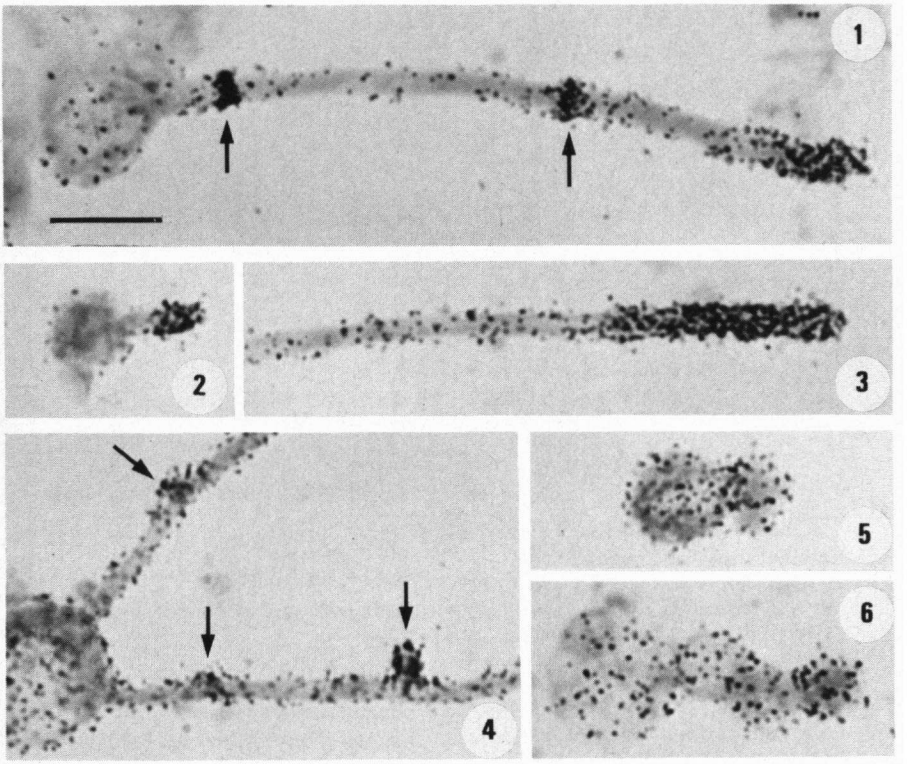
* expressed as percentage of total incorporation (dpm)

3.2. Light microscopic autoradiography

Figs. 1–6 demonstrate the pattern of incorporation of N-(acetyl-^{3}H)-glucosamine in chain-cells and in hyphae of reverted protoplasts. Hyphae exhibited a highly localized incorporation of label at the apex, although there was some variation in the length of the tip region showing this strong labelling (*figs. 1–3*). Subapical regions showed a low incorporation. Developing septa (*fig. 1*: arrows) and lateral branches (*fig. 4*: arrows) were also marked by a strong incorporation of label. In primary cells and in chain-cells the label was uniformly incorporated after both 3 h (*fig. 5*) and 9 h (*fig. 6*) of regeneration. Even in the older, often larger, comparents of the chain-cells (*fig. 6*) and in the primary cells of reverted protoplasts with fully developed hyphae (*figs. 1* and *4*) there was still a considerable, dispersed, incorporation of label.

3.3. Electron microscopic autoradiography

Examination of electron microscopic autoradiographs, after labelling with N-(acetyl-^{3}H)-glucosamine, revealed that a major part of the silver grains was located near the cell wall (*figs. 7* and *8*). Relatively few grains were found outside the wall and over the cytoplasm. In both primary cells (*fig. 9A*) and hyphae of reverted protoplasts (*fig. 9B*) the grain density was highest just outside the plasmalemma, i.e. over the inner portion of the compact inner wall layer. In primary cells there were only few grains over the fluffy coat of the wall. Assuming that the middle of the highest column of the histogram represents the middle of the source, the half distance (HD) values for N-acetylglucosamine incorporation were determined on both sides of each histogram (*table 2*). These values were clearly lower than the HD value for a non-biological line source (width about 50 nm; SALPETER et al. 1969). Theoretical grain-density curves were fitted to both sides of each histogram, using the calculated HD values of *table 2*. These curves were constructed using the equation $f_x = f_0/(1 + x^2/d^2)$ (SALPETER et al. 1969), where f_x = grain density at a horizontal distance x from the source, f_0 = grain density at $x = 0$, and d = HD. The f_0 value was calculated by integration of f_x over the entire range of x covered by the histogram and equating the integral to the total number of grains (cf. GUPTA et al. 1973). *Fig. 9* shows that the theoretical curves fit the experimental data of the histograms. Both histograms showed some asymmetry such that

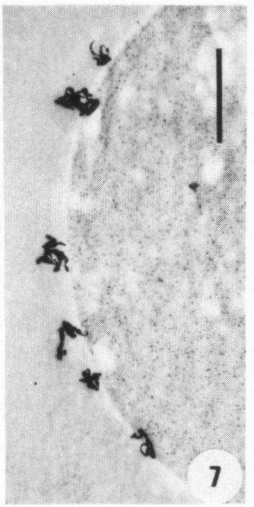


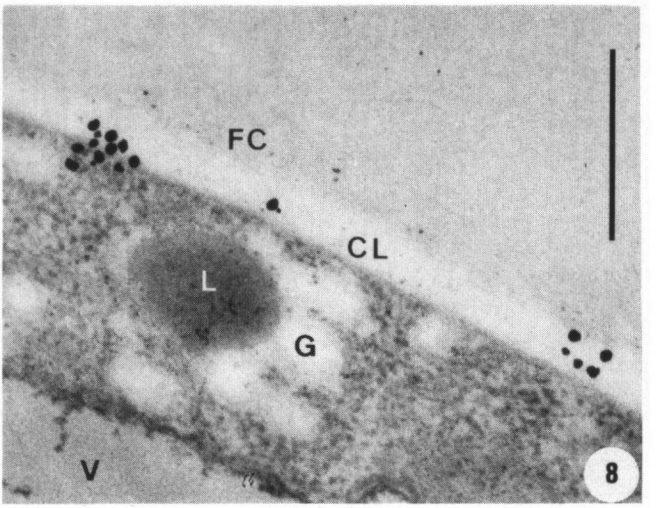
Figs. 1–6. Light microscopic autoradiographs of reverting protoplasts (figs. 1–4: 9 h of regeneration) and chain-cells (fig. 5: 3 h of regeneration; fig. 6: 9 h of regeneration) after labelling with N-(acetyl-^{3}H)-glucosamine for 30 min. All pictures are printed at the same magnification. Scale line = 10 μm.
Fig. 1. Hyphae show a strong apical incorporation of label. Subapical hyphal regions and primary cells show a lower incorporation except for developing septa (arrows) which are also strongly labelled.
Fig. 2. Hyphal initials are marked by a strong incorporation.
Fig. 3. The length of the hyphal tip region showing strong incorporation varies (compare with *figs. 1* and *2*).
Fig. 4. Lateral hyphal branches are marked by a strong incorporation of label (arrows).
Figs. 5 and 6. Chain-cells show a uniform incorporation of label.

the decrease of the grain density was more rapid over the inside than over the outside of the cell (*fig. 9*; *table 2*).

4. DISCUSSION

Labelled N-acetylglucosamine, when added to regenerating protoplasts of *Schizophyllum commune*, is incorporated in both chitin and the R-glucan complex of the wall. Since the R-glucan complex contains both glucosamine and





Figs. 7 and 8. Electron microscopic autoradiographs of primary cells after labelling with N-(acetyl-^{3}H)-glucosamine for 30 min, showing silver grains over the cell wall.
Fig. 7. Kodak D-19 development. Scale line = 1 μm.
Fig. 8. GEA development. CL = compact inner wall layer; FC = fluffy coat of the wall; G = glycogen; L = lipid; V = vacuole. Scale = 0.5 μm.

Table 2. The HD values in electron microscopic autoradiographs after labelling with N-(acetyl-^{3}H)-glucosamine for 30 min (data from *fig. 9*). The HD values were determined from the middle of the highest column on both sides of the histogram. For comparison the HD values for a line source of 50 nm width are given (SALPETER et al. 1969).

Object	HD value (nm)*	
	inside	outside
primary cells	74	84
hyphae	67	95
line source (50 nm)	130	130

* The HD value is a measure for the resolution of the employed autoradiographic method and is defined as the distance from a radioactive line source within which 50% of the total number of the developed grains fall (SALPETER et al. 1969). It has been shown that for unstained sections with grey interference colors, Ilford L4 emulsion and the GEA developer, the HD value is about 130 nm (SALPETER & MCHENRY 1973).

acetylglucosamine residues (SIETSMA & WESSELS, unpublished) only part of the aminosugars was labelled using acetyl-labelled N-acetylglucosamine in the autoradiographic experiments. Nevertheless, it is clear that both chitin and the R-glucan complex contribute to the generation of silver grains.

The observed asymmetry in grain distribution in the electron microscopic

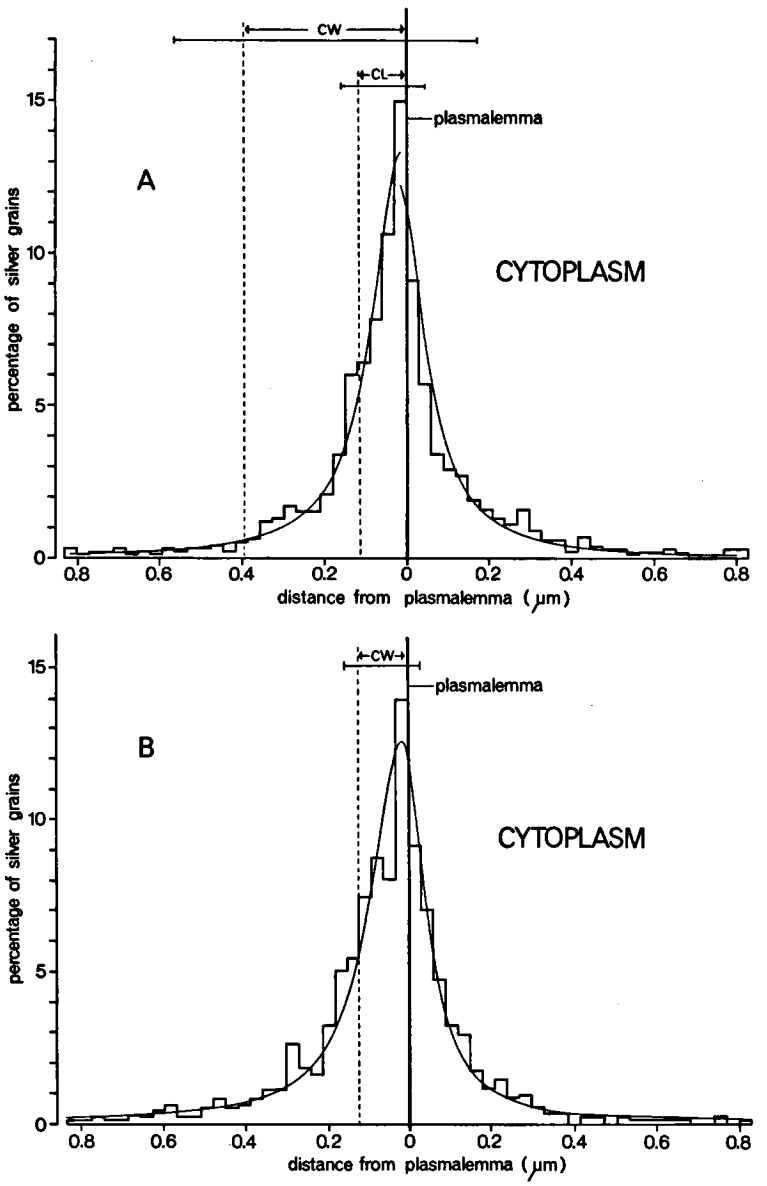


Fig. 9. Histogram of silver grain distribution in electron microscopic autoradiographs after labelling regenerating protoplasts of *Schizophyllum commune* (9 h of regeneration) with N-(acetyl-^{3}H)-glucosamine for 30 min. The mean thickness of the wall (CW) and its compact inner layer (CL) are indicated with the standard deviation of the mean. The drawn curves represent the theoretically expected grain-density distributions for a line source with HD values as given in *table 2*.

A: Primary cells. Total number of counted grains 2332.

B: Hyphae of reverted protoplasts. Total number of counted grains 1332.

autoradiographs may be related to asymmetry of the radioactive source or to a strong self-absorption of radiation on the inside of the cell, because the cytoplasm is more electron-dense than the wall and the embedding resin.

The fact that the resolution obtained in this study is apparently better than that of a non-biological line source of about 50 nm (SALPETER & MCHENRY 1973) may be due to the presence of a narrower source and/or to a strong self-absorption of radiation. Similar high resolution values for heavy metal stained biological specimens have also been obtained by CARO (1962) and GUPTA et al. (1973).

The low HD values thus indicate that the silver grains over and adjacent to the cell wall were produced by beta-particles emitted from a narrow source just outside the plasmalemma in the wall. In primary cells the radioactive source can even be located at the inside of the compact inner wall layer, which is known to consist of chitin microfibrils embedded in R-glucan matrix. Thus it appears that chitin and R-glucan are deposited in the wall at the inner wall surface only. Also for cellulose, autoradiographic evidence for apposition has been obtained (RAY 1967; see also ROLAND 1973).

The absence of alcohol-insoluble radioactive material in the cytoplasmic fraction, in which only radioactive uridine-diphosphate-N-acetylglucosamine could be identified, appears to exclude that chitin or R-glucan were synthesized in the cytoplasm and then excreted into the wall. Rather these facts support the view that these polysaccharides were synthesized at the surface of the plasmalemma. For *Saccharomyces cerevisiae* the synthesis of chitin by plasmalemma preparations has recently been demonstrated (DURÁN et al. 1975). However, for the R-glucan complex the possibility remains that the glucan part was synthesized in the cytoplasm, the hexosamine being added in the wall.

The two regeneration forms of *S. commune* protoplasts clearly differed in the pattern of incorporation of N-acetylglucosamine in the wall. Primary cells and chain-cells showed a continued disperse incorporation but the emerging hyphae were characterized by a highly localized incorporation at the hyphal apex. Such a localized incorporation of N-acetylglucosamine has earlier been observed in normal hyphae of *S. commune* (GOODAY 1971) and in other fungi (BARTNICKI-GARCÍA & LIPPMAN 1969; KATZ & ROSENBERGER 1970, 1971; GOODAY 1971).

It thus appears that the generation of a localized growth centre is an important event in the reversion of *S. commune* protoplasts. It has recently been demonstrated (VAN DER VALK & WESSELS 1976) that in such an apical growth centre chitin and R-glucan are synthesized co-ordinately as a complex. In this complex chitin microfibrils are completely embedded in R-glucan whereas in primary cells and chain-cells R-glucan only surrounds the inner chitin microfibrils of the wall.

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