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Ultrastructural Changes and Biochemical Events in Basidiospore Germination of *Schizophyllum commune*

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Electron microscopic features and biochemical events were outlined in basidiospore germination of *Schizophyllum commune*. Normal ultrastructural changes included prominent vacuolization and more abundant endoplasmic reticulum. A lag phase in outgrowth included depletion of cellular reserves of trehalose, mannitol, and arabitol and subsequent increases in ribonucleic acid and protein. Depletion of polyols required exogenous carbon and nitrogen sources and was arrested by protein synthesis antagonists. Outgrowth subsequent to the lag period was accompanied by increased glycogen deposition and alkali-soluble glucan production.

Basidiospore germination in the filamentous fungus *Schizophyllum commune* can be regarded as an elementary example of eukaryotic cytodifferentiation. Aseptate binucleate basidiospores undergo a lag phase in outgrowth followed by extensive elongation prior to nuclear division(s) and subsequent elaboration of complex basidiomycetous septa (1, 15, 22). A typical respiratory chain appears operative in the ungerminated basidiospore (21), and this correlates with the presence of conventional mitochondria, as revealed by electron microscopy (25, 38). Biochemical features of basidiospore germination in *S. commune* include the acquisition of sugar alcohol (polyol) oxidation capacity (23) and the depletion of intracellular reserves such as mannitol and arabitol (24). Moreover, a striking increase in a nicotinamide adenine dinucleotide phosphate (NADP)-dependent glutamate dehydrogenase activity occurs early in germination regardless of the sole nitrogen source in the medium, although a nicotinamide adenine dinucleotide (NAD)-coupled glutamate dehydrogenase appears depressed in activity and is only elevated during mycelial growth (6). The current investigation (Aitken and Niederpruem, Bacteriol. Proc., p. 31, 1968) delineates the sequence of changes in the levels of polyols, trehalose, and macromolecular constituents relative to changes in ultrastructure during basidiospore germination of *S. commune*.

MATERIALS AND METHODS

Cultures. Basidiospores were produced and collected from dikaryotic fruit-bodies of *S. commune* after mating compatible homokaryotic mycelia (35 hf A41B51 × 1 A51B41) as described previously (22).

Lipids and nucleic acids. Extraction procedures were based on the methods of Schmidt and Thannhauser (31) as modified for *S. commune* by Wessels (39). Lipids were extracted with ethanol, ethanol:ether (1:1, v/v), and ether. Total lipid content was quantified by the dichromate procedure (2) using palmitic acid as a standard.

The remaining precipitate was extracted with cold trichloroacetic acid (5%, w/v), and the acid-soluble material was measured spectrophotometrically at 260 nm by using base-hydrolyzed yeast ribonucleic acid (RNA; Worthington Biochemical Corp., Freehold, N.J.) as a standard.

The residue was extracted with KOH (1 N) at 37 C for 18 hr, neutralized with HCl (6 N) and acidified with trichloroacetic acid (11%, w/v). The RNA content was determined by the method of Schneider (32) and also by ultraviolet absorption (260 nm). The remaining material containing the deoxyribonucleic acid (DNA), protein, and cell wall substances was extracted with hot trichloroacetic acid (5%) at 90 C for 30 min. The DNA content was determined by absorbance at 265 nm and by the method of Burton (3).

Protein. Cells were extracted with cold trichloroacetic acid (5%), and the residue was suspended in KOH (1 N) and heated at 100 C for 10 min. The protein concentration was determined by the method of Lowry et al. (19), by using bovine albumin fraction V (Pentex, Inc., Kankakee, Ill.) as standard.

Carbohydrates and polyols. The low-molecular-weight carbohydrates were extracted with hot ethanol as described earlier (23, 24). Glycogen was extracted with acetic acid (0.5 N at 80 C) as outlined by Wessels (39). After acetic acid extraction, the residue was suspended in KOH (5%) and allowed to stand at 25 C (±3) for 18 hr. The supernatant solution contained the

alkali-soluble polysaccharide (S-glucan), and the residue contained the alkali-insoluble carbohydrate (R-glucan). The S-glucan is predominantly α-1,3 linked, whereas the R-glucan contains β-1,3 and β-1,6 bonds among its constituents (25).

Total carbohydrate was determined by the anthrone method (8). Polyol was analyzed by Faulkner's modification (9) of the chromotopic acid procedure of West and Rapoport (41) by using mannitol as standard. Ketose was measured by the cysteine-carbazole method of Dische and Borenfreund (7) with fructose as standard, and glucose was measured by the glucose oxidase method (Worthington Biochemical Corp.). Total reducing sugar was evaluated by the anthrone procedure with glucose as standard, and trehalose was estimated by difference after subtracting glucose and fructose as determined above.

Carbohydrates and polyols were identified by descending paper chromatography (24). The sugars were revealed by the alkaline silver nitrate dip (35), and polyols were revealed by a periodate-benzidine dip (11).

Electron microscopy. Spores were collected from 1- to 2-day sheddings by adding fixative to petri dish lids containing the spores. The suspension was passed through membrane filters (0.8 μm pore size; Millipore Corp., Bedford, Mass.), and fresh fixative was added to the cells on the filter. Spores collected in distilled water were used to inoculate 100 ml of minimal broth in flasks which were then shaken at 26 C for 4 hr. Fixative was added to the flasks; the cells were filtered (Millipore membrane filters) and then suspended in fresh fixative. Initial fixation was for 1 hr at room temperature with either 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (*p*H 7.2) or in a 1% formaldehyde (made by dissolving paraformaldehyde at 60 to 70 C in the presence of 0.1 M NaOH)-1% glutaraldehyde mixture in 0.1 M sodium cacodylate buffer containing $CaCl_2$ (0.5 mg/ml). After buffer rinses, the cells were postfixed for 2 hr in 1% buffered OsO_4, rinsed in buffer and then in distilled water, and stained from 2 to 4 hr in 0.5% aqueous uranyl acetate. Dehydration was in a graded series of ethanol followed by acetone. The material was embedded in Araldite 6005 (29) and polymerized at 60 C in a nitrogen atmosphere for 2 days. Blocks were sectioned on a Porter-Blum MT-2 ultramicrotome by using a diamond knife. The sections were mounted on collodion-methacrylate-coated grids (26), poststained for 10 min in lead citrate (28), and examined in a Philips EM/200 at 60 kv. The cells shown in Fig. 1 to 5 were fixed with 2% glutaraldehyde and that in Fig. 6 was fixed with the formaldehyde-glutaraldehyde mixture.

RESULTS

Ultrastructural features of germination. Ungerminated basidiospores of *S. commune* were binucleate with each nucleus containing a nucleolus (Fig. 1). Lipid droplets, mitochondria, membranous cisternae, vesicles, vacoules, and free ribosomes were characteristic. Endoplasmic reticulum was sparse or absent at this stage. The vesicles were of two general types: (i) vesicles approximately 80 to 90 nm in diameter of the same type, but somewhat smaller than those found in hyphal apices of this (100 to 135 nm; Heintz and Niederpruem, *unpublished data*) and other fungi (10, 12) and (ii) microvesicles approximately 25 to 35 nm in diameter (Fig. 2). Although cells shaken in broth for 4 hr generally showed no signs of germinating when examined by phase-contrast microscopy, their ultrastructural profiles had changed by this time (Fig. 3 to 5). Endoplasmic reticulum was observed more frequently and at times was the only membrane, except for its nuclear envelope homologue, which stained densely (Fig. 3). Small vacuoles with dense amorphous inclusions were a usual feature of 4-hr germinants (Fig. 4). In cells which had begun to elongate at this time, inclusion-containing vacuoles were more numerous (Fig. 5). The size of the vacuoles and the inclusions increased until some of these inclusions occupied a considerable volume of the cell (Fig. 6).

Kinetics of basidiospore germination. Cytological evaluation of early events in basidiospore germination of *S. commune* is difficult because no conspicuous germ tube pore or cap occurs in these propagules. Consequently, prior measurements of cell length, turbidity, or dry weight in glucose-asparagine minimal medium revealed a conspicuous lag phase in outgrowth (22). To discern physiological antecedents of cell elongation, a glucose-$(NH_4)_2SO_4$ broth containing one-tenth the normal phosphate buffer concentration was employed, and *p*H measurements of the culture filtrate were compared to absorbancy changes which are related to cellular dry weight (22). During the 8-hr lag phase prior to outgrowth (Fig. 7), there occurred a marked decrease in broth *p*H followed at 12 hr by increased absorbancy due to cell elongation. Neither of these events were seen when the sole carbon and nitrogen sources were withheld. Thus early assimilation of ammonia may explain these initial *p*H changes and correlates with the previously described increase in specific activity of the NADP-coupled glutamate dehydrogenase in germination of *S. commune* (6).

Carbohydrates and polyol reserves in germination. Mannitol and arabitol comprise endogenous reserves in the ungerminated basidiospores of *S. commune* (24), together with appreciable amounts of trehalose and some "glycogen." The presence of trehalose was substantiated by descending paper chromatography, Dowex-1 column chromatography by the procedure of Vining and Taber (37) and subsequent gas chromatography of the trimethylsilyl ether derivative.

Insight into roles for polyols and trehalose in

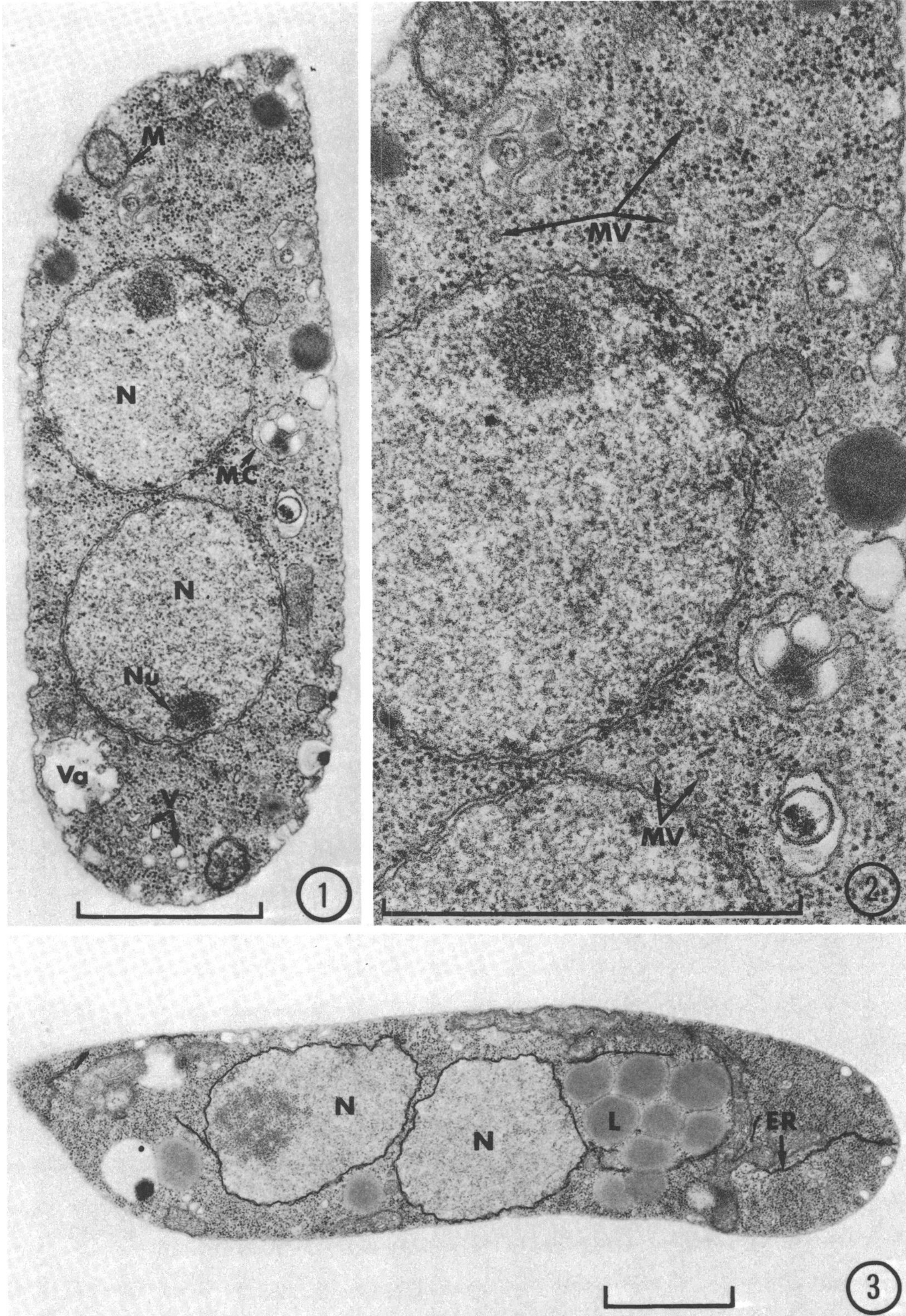
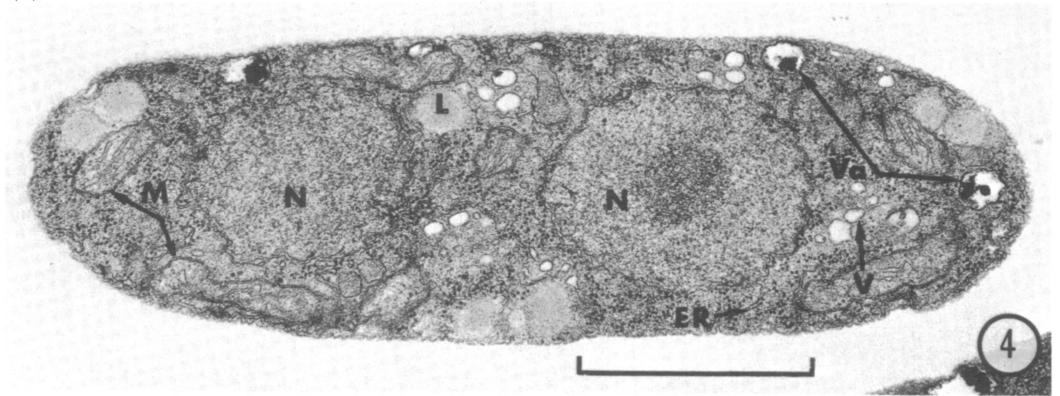
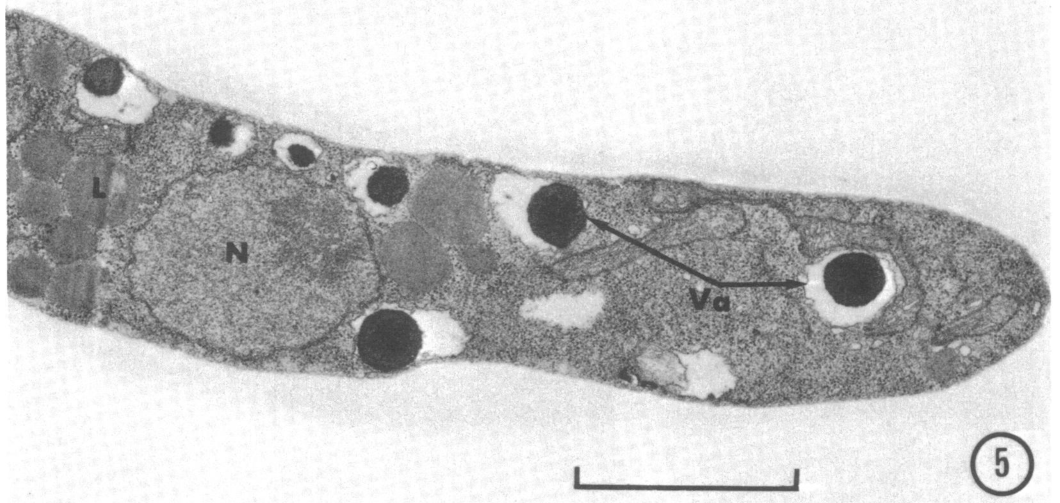


FIG. 1. *Ungerminated basidiospore of Schizophyllum commune containing two nuclei (N) with nucleoli (Nu), mitochondria (M), a membranous cisterna (MC), vacuoles (Va), and two types of vesicles (V). The larger vesicles are similar to those found in hyphal apices. In all electron micrographs, scale line indicates 1 μm.*

FIG. 2. *Higher magnification of a portion of Fig. 1 showing the microvesicles (MV) and details of the protoplasm.*

FIG. 3. *Four-hour germinant showing densely stained endoplasmic reticulum (ER) and nuclear envelope as compared to other, more lightly stained, membranous components. Nuclei (N), lipid (L).*





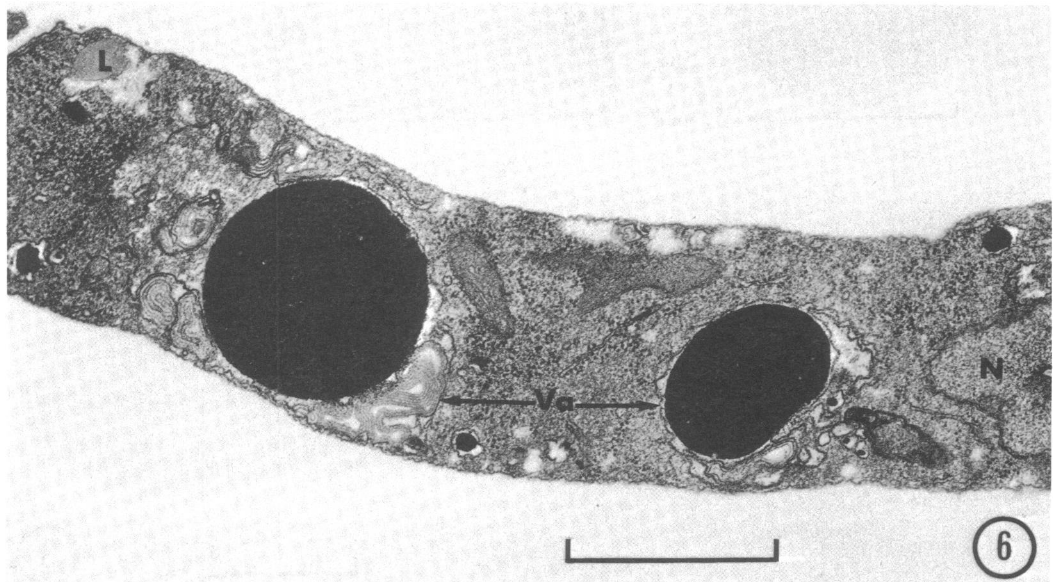


FIG. 4. *Four-hour germinant with small vacuoles (Va) containing dense, amorphous material, nuclei (N), endoplasmic reticulum (ER), mitochondria (M), lipid (L), and hyphal tip vesicles (V) in zones of exclusion.*

FIG. 5. *Elongated germinant in which the numbers and size of the vacuoles with inclusions (Va) have increased.*

FIG. 6. *Vacuoles (Va) contain large dense inclusions and membranes. The inclusions occupy half or more of the hyphal diameter. Small vacuoles with similar dense inclusions are associated with the large inclusion-containing vacuoles.*

cytodifferentiation came from studies of these substances during germination in standard glucose-asparagine minimal broth (Fig. 8). A marked decrease in total polyols and trehalose occurred during the lag phase of germination and was subsequently followed by an accumulation of these constituents. Glycogen content remained essentially constant during the lag phase of outgrowth and only increased later (Fig. 8). Both pool depletion and germination required exogenous carbon and nitrogen sources; neither germination nor decreases in polyols or trehalose occurred in minimal broth lacking glucose and asaparagine.

Kinetic studies showed that pool depletion occurred during the first 4 hr of the lag period. Chromatographic analysis showed that arabitol disappeared over this interval although some mannitol persisted. Beyond the lag period, intracellular pool components increased again (Fig. 8), and additional substances including glucose, fructose, and glycerol were apparent. Therefore, preferential utilization of endogenous polyols and trehalose rather than glycogen stores is an apparent antecedent of outgrowth whereas glycogen accumulation appears more important during cell elongation.

The nature of control devices operative in polyol depletion during germination was elucidated by inhibitor studies. The effects of cycloheximide (20 μg/ml) and fluorophenylalanine (30 μg/ml) on polyol changes in basidiospores incubated in glucose-asparagine broth are shown in Figure 9. Spore germination was arrested, and no depletion of polyol reserves occurred in the presence of either poison but a substantial increase in polyol (as mannitol) was observed in the case of cycloheximide.

Cell wall components in germination. Specific

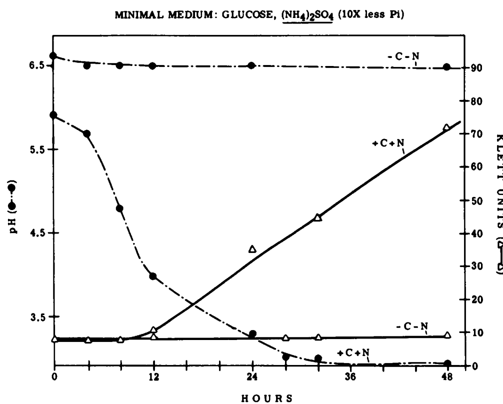


FIG. 7. *Changes in broth pH and turbidity during germination in glucose-$(NH_4)_2SO_4$ medium containing one-tenth standard phosphate buffer.*

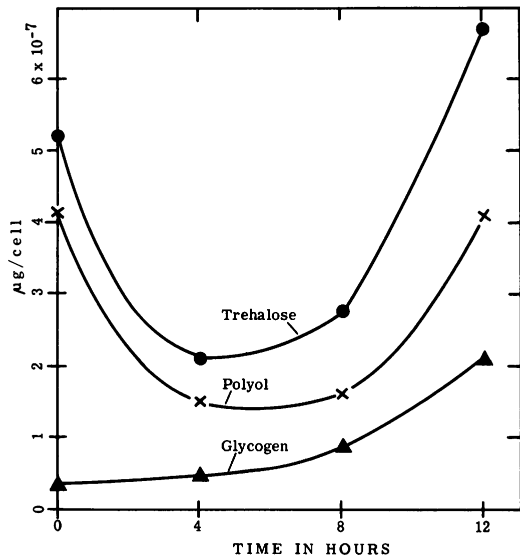


FIG. 8. *Comparison of changes in carbohydrate reserves during basidiospore germination in glucose-asparagine broth.*

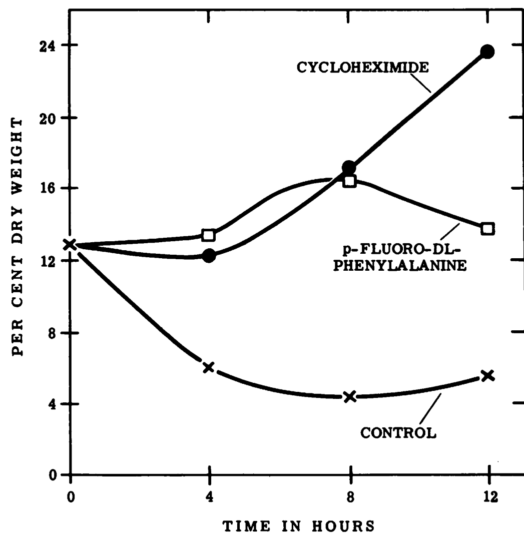


FIG. 9. *Effects of protein synthesis inhibitors on changes in total polyols during germination. Final concentration: cycloheximide (20 μg/ml); p-fluoro-DL-phenylalanine (30 μg/ml).*

glucans, distinguished by their solubility in alkali (KOH, 1 N), comprise a significant proportion of the cell wall of *S. commune* (39). Accordingly, initial studies dealt with the ratio of S-glucan/R-glucan in basidiospores and germlings of *S. commune*. Although the S/R ratio of ungerminated basidiospores was considerably less than unity

(0.41), a marked shift in the ratio of S-glucan/R-glucan was evident after 16 or 30 hr of germination, with S/R values of 1.30 and 1.52, respectively. Cultures lacking carbon or nitrogen sources failed to show the three- to fourfold increases in S/R values and failed to germinate. This increase in S/R value in normal germination may reflect preferential synthesis of S-glucan or selective degradation of R-glucan during outgrowth. The former idea is favored because the hydrolytic enzyme R-glucanase is currently believed to be repressed in homokaryotic mycelium of *S. commune* (40).

Kinetic studies of germination changes in cell wall components were compared to glycogen reserves (Fig. 10). Although R-glucan decreases between 12 and 16 hr, a more conspicuous increase in S-glucan occurs from 4 hr until 20 hr of germination. Therefore, R-glucan degradation may partially explain the prominent increase in the S/R ratio of cell wall components, but S-glucan biosynthesis appears to be primarily responsible for these changes during outgrowth.

Lipids in germination. Electron microscopic studies revealed abundant lipid vacuoles in germlings of *S. commune*, and their presence was correlated with phase-contrast microscopic observations showing increased vacuolation in live germinants. Although total lipid measurements proved variable during various stages of germination, a three-fold increase in total lipid content was consistently observed in 12-hr germlings of *S. commune* which had arisen in glucose-asparagine broth.

DNA, RNA, and protein in germination. The free nucleotides did not change appreciably in concentration. Changes in the content of DNA, RNA, and protein were evaluated during basidiospore germination (Fig. 11). The constant level of

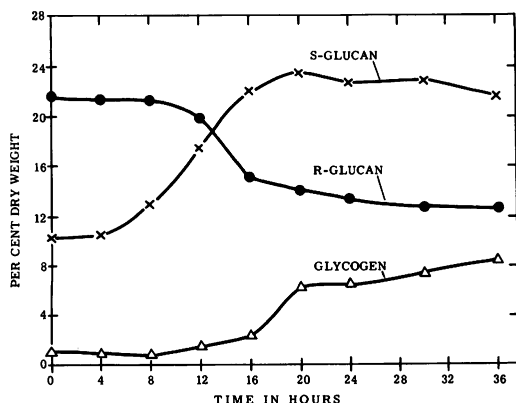


FIG. 10. *Changes in cell-wall glucans relative to "glycogen" in basidiospore germination.*

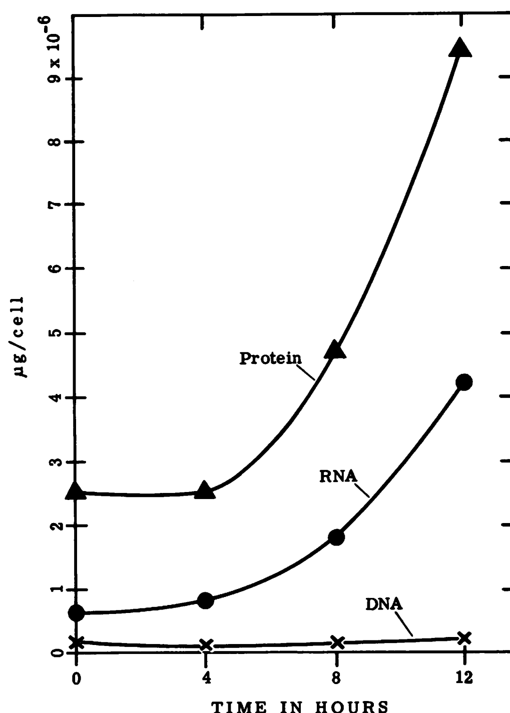


FIG. 11. *Changes in protein, RNA, and DNA in germination.*

DNA during the lag phase was to be expected because cytological observations showed that nuclear division is delayed until considerable elongation occurs in basidiospores of *S. commune* (1, 15). In contrast, RNA, and protein levels increased shortly after the spores were immersed in broth, although at least 4 hr elapsed before these changes were evident. Also of interest are data showing that nearly parallel increases in RNA and protein preceded outgrowth but were subsequent to the major depletion of polyols and trehalose.

DISCUSSION

The present work reveals temporal aspects of biochemical events in basidiospore germination of *S. commune*. A lag phase preceding outgrowth can now be considered a period of active metabolism when intracellular reserves such as trehalose and especially arabitol are rapidly depleted, followed by general increases in both RNA and protein. Glycogen accumulation and conspicuous changes in cell wall glucans occur during outgrowth. As expected, DNA content appears unchanged during cell elongation, and precocious nuclear division has never been observed in the binucleate basidiospore of *S. commune*.

The widespread occurrence of polyols and tre-

halose in dormant systems (18) attests to the general importance of these reserves in development although control devices governing their metabolism remain obscure. A focal point from which to approach these issues is spore germination in fungi. The hydration of fresh rather than aged uredospores of *Puccinia graminis tritici* leads to decreases in trehalose, mannitol, and arabitol (42). In contrast, basidiospores of *S. commune* do not mobilize these reserves in aqueous medium unless exogenous carbon and nitrogen sources are present. Moreover, depletion of polyols in *S. commune* is sensitive to protein synthesis antagonists. Similarly, early utilization of trehalose prior to germ tube emergence in *Myrothecium verrucaria* also required exogenous substrates (20). In yeast, also, trehalose is consumed in the lag phase of growth, provided that exogenous glucose is present (27).

It is important to note that the fate of metabolized trehalose or polyols remains unknown in most of these situations. Thus, although the activation or de novo origin of trehalase or polyol dehydrogenases could be involved, other explanations are also plausible. These include the emergence of ancillary enzymes of carbohydrate metabolism and the possibility that pool depletion involves an excretion of these reserves. Support for the latter has been provided in studies of development in *P. graminis tritici* (5) and *Sclerotinia sclerotiorum* (4). The alternate view that trehalase activity is involved in pool depletion is substantiated in germination of *Neurospora tetrasperma* (33), *Aspergillus oryzae* (14), and in the budding cycle of yeast (16).

A general restriction of protein synthesis in the ungerminated basidiospore of *S. commune* was unexpected because these propagules contain abundant ribosomes as revealed by electron microscopy. Moreover, the spores readily incorporate ^{14}C-labeled amino acids into trichloroacetic acid-precipitable material, and the incorporation was sensitive to cycloheximide (25 μg/ml; 21). Measurements of both RNA and protein showed early, though not immediate, increases during the lag phase of outgrowth, and these data correlate with rapid assimilation of ammonia and increased specific activity of the NADP-coupled glutamate dehydrogenase (6). Recent studies of ribosomes isolated during basidiospore germination of *S. commune* indicate that monosomes predominate in spores although a different class of polysomes occur in 6-hr germlings (17). The appearance of this latter species of polysomes may explain the 4-hr lag period before general protein content increases during *S. commune* germination in the present work. In contrast to these differences, aminoacyl-transfer RNA appears similar in spores or germlings of *N. crassa* (13), *A. oryzae* (34), *Botryodiplodia theobromae*, and *Rhizopus stolonifer* (36). In harmony with these latter observations is the situation encountered in basidiospores of *Lenzites saepiaria*, in which incorporation studies employing labeled precursors of RNA and protein showed that macromolecular synthesis was immediate although DNA synthesis occurred later (30).

The increase in cell wall S-glucan of *S. commune* during outgrowth cannot be explained by R-glucan degradation and glucose recycling initially because the S-glucan level increases almost twofold before R-glucan content diminishes. This period of cytodifferentiation may provide an opportune time to examine the nature and control of S-glucan synthetase in *S. commune* development.

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