Physiological and Biochemical Changes Associated with Macroconidial Germination in *Microsporum gypseum*

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A study was made of the metabolic processes associated with macroconidial germination in *Microsporum gypseum*. The optimum conditions for stimulation of endogenous respiration, changes in chemical composition as germination proceeds, and the uptake and synthetic fates of amino acids, glucose, and uracil were investigated. The assimilation and conversion of \(^{14}\text{C-glucose, }^{14}\text{C-amino acids, and }^{14}\text{C-uracil into the cell pool and into trichloroacetic acid-precipitable material were studied during the early stages of germination (i.e., prior to germ-tube emergence). The macroconidia were not metabolically inert for any significant period of time after exposure to germination conditions. Rather, the spores rapidly assimilated all metabolites and slowly converted them into macromolecules. Investigations of the effect of inhibitors of nucleic acid and protein synthesis prior to germ-tube emergence and during early germ-tube elongation suggested significant changes in metabolism and cell permeability may be correlated with the emergence of germ tubes. Radioactivity of incorporated glucose was found to be associated largely with the lipid fractions of the macroconidia early in germination.

The changes associated with spore germination in fungi are less well understood than are the corresponding events in bacteria (23). Recent investigations have been concerned with the germination of the zoospores of fungi (13, 21), the conidia of *Aspergillus* (8, 26), and certain plant pathogens (2, 7, 22). However, very little is known about the situation in the majority of the fungi, including the dermatophytic fungi. Since the macroconidia of these organisms (at least under some conditions) may represent the infective agent, a study of the physiological and biochemical processes involved in their germination is of interest.

The fate of exogenous metabolites during early macroconidial germination (prior to germ-tube emergence), has not been well documented in *Microsporum gypseum*. One previous study (1) suggests that the uptake of small molecules may commence immediately after suspension of the macroconidia in germination medium. In contrast, a considerable lag period has been reported in *Aspergillus* prior to rapid assimilation of \(^{14}\text{C-glucose (8).}

The present study was undertaken to define the optimal conditions for stimulation of macroconidial respiration, to investigate the uptake and assimilation of glucose, amino acids, and uracil, to assess the changes in the chemical composition of macroconidia during early germination, and to determine the effects of inhibitors of protein and nucleic acid synthesis upon the morphological and synthetic processes accompanying germination.

MATERIALS AND METHODS

Organism and growth conditions. The origin, growth, and sporulation characteristics of the strain of *M. gypseum* which was utilized have been described previously (9). Sporulation conditions and spore purification also have been described elsewhere (10).

All the experiments described here were carried out in germination medium (0.35 mg of glucose per ml, 0.25 mg of neopeptone per ml) at 37°C. This concentration of nutrients was chosen because preliminary experiments on the stimulation of endogenous respiration indicated that use of these concentrations provided maximal stimulation. This medium also had the virtue of providing a reproducible developmental sequence in a time period which was amenable to

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experimental investigation. The concentration of spores, isotopes, and inhibitors used varied, and specific conditions are given for each experiment. For uptake studies, 7-day-sporeulated Roux flask cultures were cooled to 4 C. All harvesting and purification procedures were carried out at 4 C. Purified spores were equilibrated at 37 C for 5 min prior to addition to the germination medium.

**Manometric techniques.** Oxygen consumption was measured by standard Warburg methods (24), using single side-arm flasks. Glucose and neopeptone (Difco) were contained in the side-arm and added after 5 min of preincubation at 37 C.

**Uptake studies.** Macroconidial germination was initiated as described previously (9, 10). The incorporation of 14C-metabolites into whole macroconidia, trichloroacetic acid-insoluble material, and pool material was determined by the filtration method of Britten and McClure (3). Whole-cell counts were obtained by filtering macroconidia (5 mg, equivalent to 10^8 spores per ml) onto 1.2-μm pore size filters (Millipore Corp., Bedford, Mass.), in an E8B precipitation apparatus (Tracelab, Waltham, Mass.). Conidia were washed with a 10x sample volume of germination medium at 37 C. A duplicate sample (5 mg, containing 10^8 spores per ml) was combined with an equal volume of ice-cold 10% trichloroacetic acid and extracted at 0 C for a minimum of 30 min. Acid-insoluble material was collected by filtration (as above), and washed with a 10x sample volume of ice-cold 5% trichloroacetic acid. Dried filters were placed in vials containing 5 ml of scintillation fluid (Liquifluor, New England Nuclear Corp., Boston, Mass.), and counted in a liquid scintillation spectrometer (Nuclear Chicago Corp., model 725, Des Plaines, Ill.). Preliminary experiments indicated that aminoaacetyl-transfer ribonucleic acid molecules represented an insignificant part of this fraction, and, consequently, hydrolysis of these molecules by heating was not routinely performed.

**Inhibitor studies: effect on continuous incorporation of metabolites by germinating macroconidia.** Mature macroconidia were suspended in germination medium at a concentration of 10^8 spores per ml. Isotope (at a level of 1 μCi/ml) and the inhibitors (at various concentrations) were added at the times indicated in the figures and tables. At various time intervals, 100-μl samples were withdrawn, and the incorporation of 14C-uracil and 14C-amino acids into acid-insoluble material was determined as described above for the uptake studies. Background counts (those retained by the filters in the absence of cells) have been subtracted from the reported counts for each experiment. For consistency and to conform with the data on chemical composition, the radioactive counts for these experiments are expressed on the basis of 10^8 macroconidia.

**Effect on morphological changes associated with macroconidia germination.** Macroconidia were allowed to germinate, as usual in the presence of either inhibitor, and counts of the number of germinated spores were made, using a Petroff-Hauser counting chamber.

**Chemical fractionation of macroconidia.** Macroconidial constituents (50-mg samples) were fractionated by the method of Roberts et al. (19), as modified by Clifton and Sobek (5). Samples of the fractions were plated onto stainless steel planchets, dried, and counted in a thin end-window Geiger tube attached to a Nuclear Chicago model 181A scaler equipped with an automatic gas-flow counter.

**Analytical determinations.** Dry weights were determined by filtering samples containing 10^4 macroconidia per ml through pre pared 0.3 μm filter discs (Millipore Corp.). The spores were washed twice on the filters with physiological saline and dried to a constant weight.

Nucleic acids and proteins were extracted from macroconidial suspensions by a modification of the method of Neidhardt and Magasanik (15). Macroconidia were ruptured by freeze-thawing, followed by homogenization in a glass tissue homogenizer fitted with a Teflon homogenization pestle. The ruptured spore fractions were precipitated with cold trichloroacetic acid (10% w/v), extracted with cold trichloroacetic acid (5% w/v) at 4 C overnight, and washed twice with cold trichloroacetic acid (5% w/v). The cold trichloroacetic acid extract and washes were pooled, and the residue was reextracted with hot trichloroacetic acid (5% w/v) at 70 C for 30 min, three times; the hot trichloroacetic acid extracts were likewise pooled. The residue was dispersed with the tissue homogenizer and extracted successively with 0.1 N NaOH at room temperature overnight, followed by 0.5 N NaOH at 90 C for 30 min, and then with 1.0 N NaOH at 90 C, until no more Lowry-positive material could be extracted. The alkaline extracts were analyzed for protein by the Folin reaction (14), using crystalline bovine serum albumin as a standard. The trichloro acetic acid extracts were analyzed for nucleic acids. Deoxyribonucleic acid (DNA) was measured by the diphenylamine method described by Burton (4), using calf thymus DNA as a standard. Ribonucleic acid (RNA) was determined by the orcinol reaction (6), with yeast RNA as a standard. All reported values represent the averages from two to four independent determinations.

**Chemicals.** Crystalline bovine serum albumin, yeast RNA, and calf thymus DNA were obtained from Sigma Chemical Co., actinomycin D from Calbiochem, and cycloheximide (Actidione) from Upjohn Company. The radiochemicals used in the uptake experiments were purchased from Schwarz Bio Research, Orangeburg, N.Y. Specific activities were B-glucose-U-14C (250 mCi/m mole); protein hydrolysate-U-14C (1 mCi/ml), and uracil-2-14C (50 mCi/m mole). Those used in the inhibitor experiments were purchased from Amersham-Searle. Specific activities were uracil-2-14C (62 mCi/m mole); amino acid hydrolysate-U-14C (63 μCi/ml).

**RESULTS**

Under the conditions used in these experiments, *M. gypseum* macroconidia changed little in appearance for the first 3 hr after they
were placed into germination medium (Fig. 1A). Between 3 and 4 hr they began to form germ tubes (Fig. 1B), and by 18 hr each spore possessed multiple germ tubes which imparted a spider-like appearance to the young germings (Fig. 1C).

Changes in macromolecular cell constituents during early germination. Values for dry weight, RNA, and protein showed slight changes during the earliest stages of germination (Fig. 2), but all showed significant changes prior to germ-tube emergence (Fig. 2 and Table 1). The dry weight, RNA, and protein increased slightly between 120 to 180 min of germination and then decreased prior to germ-tube formation. DNA began to increase sharply after the initiation of germination.

Stimulation of spore respiration. Figure 3 depicts the effect of additions of varying amounts of glucose and neopeptone on spore respiration: 0.33 mg of glucose per ml and 0.25 mg of neopeptone per ml provided maximal stimulation. Addition of higher concentrations of either metabolite had no further stimulatory effect on $O_2$ consumption.

Uptake of $^{14}$C-glucose, $^{14}$C-amino acids, and $^{14}$C-uracil. During early spore germination (prior to germ-tube formation), $^{14}$C-glucose (Fig. 4), $^{14}$C-amino acids (Fig. 5), and $^{14}$C-uracil (Fig. 6) were rapidly taken up by the macroconidia and then were slowly converted into acid-precipitable material. In all the uptake experiments, the zero-time point represents approximately 10 sec of incorporation due to the time lag between sampling and filtration.

Owing to the high external concentration of amino acids, it would seem unlikely that much of the $^{14}$C-glucose would be required for protein synthesis. Hence, it was of interest to know what type of macromolecules were synthesized from exogenous glucose. Table 2 lists the composition of fractionated acid-insoluble material which accumulated during early macroconidial germination. The majority of the label was localized in the acid alcohol-soluble fraction.

Effect of inhibitors of nucleic acid and protein synthesis on the germination process. Prior to germ-tube emergence, actinomycin D, at concentrations ranging from 5 to 100 $\mu$g/ml, had little effect on either the incorporation of $^{14}$C-uracil into acid-precipitable material (Fig. 7) or the process of germination (Table 3). Concentrations above 100 $\mu$g/ml did produce morphologically abnormal spores. However, these high levels were not considered useful since metabolic processes other than macromolecular syntheses were affected, i.e., glucose uptake (unpublished data).

During the time immediately preceding germ-tube emergence (Fig. 7) and during the process of germ-tube elongation (Tables 4 and 5), actinomycin D did inhibit the incorporation of $^{14}$C-uracil and $^{14}$C-amino acids into acid-precipitable material.

Although macroconidia produced germ tubes in the presence of actinomycin D, these germ tubes failed to elongate, and the macroconidia never attained the spider-like appearance which characterizes normal germlings (Fig. 1C).

In contrast, cycloheximide, at a concentration of 10 $\mu$g/ml or greater, inhibited the incorporation of $^{14}$C-amino acids into trichloroacetic acid-precipitable material prior to the onset of germ-tube emergence (Fig. 8; Table 6). Likewise, this concentration of cycloheximide inhibited germ-tube formation (Table 3). Cycloheximide was less effective in inhibiting macromolecular synthesis in the later stages of germination.

DISCUSSION

On the basis of the uptake and incorporation studies presented, it is clear that macroconidia of M. gypseum were able to assimilate glucose, amino acids, and uracil immediately after exposure to germination-inducing conditions and to convert these metabolites slowly into macromolecules. This agrees with the findings of Barash et al. (1), who reported that labeled uridine, thymine, and leucine were incorporated into M. gypseum macroconidia within 5 min of incubation in germination medium. Barash et al. (1) concluded that all the enzymes required for macromolecular synthesis were present in the resting spore and that M. gypseum does not show a lag period prior to synthesis as is true for many bacteria (23) and for the conidia of Aspergillus (26).

However, the decreases in dry weight, protein, and RNA values which we have found associated with the earliest stages of germination suggest that there may be considerable macromolecular turnover accompanying the observed de novo synthesis. Moreover, the measurements of the accumulation of $^{14}$C-metabolites into intracellular pools indicate that, prior to germ-tube emergence, the macroconidia were not capable of maintaining constant pool levels of small molecules. That is, after an initial uptake period, the intracellular pools failed to stabilize. Also, in the case of uracil and amino acids, only a small fraction of the total external radioactivity was present in the cells, even after 2 hr of incubation. At
Fig. 1. Macroconidia of *M. gypseum*, Zeiss/Nomarski differential interference microscopy, unstained preparations. Bar represents 10 μm. A, Mature macroconidia prior to initiation of germination. B, macroconidia 4 hr after initiation of germination. Germ tubes are just beginning to emerge. C, macroconidia 18 to 24 hr after initiation of germination. Note presence of multiple germ tubes, imparting a spider-like appearance to these germlings.
MACROCONIDIAL GERMINATION IN M. GYPSEUM

**Table 1. Changes in chemical composition of M. gypseum macroconidia upon germination**

| Morphological state                              | Dry weight (mg) | DNA (µg) | RNA (µg) | Protein (µg) |
|--------------------------------------------------|-----------------|----------|----------|-------------|
| Mature macroconidia prior to initiation of germination (zero time) | 12.7            | 10       | 1,609    | 5,310       |
| Germ-tube emergence (4 hr)                       | 10.7            | 83       | 1,361    | 4,108       |

*Germination system contained: macroconidia, 10⁹/ml; glucose, 0.33 mg/ml; and neopeptone, 0.25 mg/ml.

*All values based on 10⁹ macroconidia.

Present, we can offer no satisfactory explanation for these results since the observed decrease in pool levels could be due to efflux, membrane permeability changes, changes in pool size due to turnover, or a combination of...
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precipitable acid-precipitable roconidia, macroconidia. Germination 0.25 mCi/ml; "4C-protein counts; ml. ble roconidia.

conidia, 0.25 mCi/ml; "4C-glucose, 0.33 mg/ml; and neopeptone, 0.25 mg/ml. Symbols: total counts; Δ, acid-precipitable counts; O, pool counts (total counts – acid-precipitable counts).

these possibilities. A much more detailed study will be necessary before we can decide between these alternatives.

If the absence of de novo synthesis during early germination were true, M. gypseum would resemble more closely theuredospores of certain plant rusts (20) than the conidia of Glomerella cingulata, Neurospora sitophila, and Aspergillus niger, in which protein synthesis occurs during germination (22). However, the fact that cycloheximide inhibits both germ-

FIG. 5. "4C-amino acid uptake by germinating macroconidia. Germination system contained: macroconidia, 10 mg/ml (equivalent to 10³/ml); 1 μCi of "4C-glucose per ml (specific activity, 1 mCi/ml); "4C-glucose, 0.33 mg/ml; and neopeptone, 0.25 mg/ml. Symbols: total counts; Δ, acid-precipitable counts; O, pool counts (total counts – acid-precipitable counts).

Fig. 6. "4C-uracil uptake by germinating macroconidia. Germination system contained: macroconidia, 10 mg/ml (equivalent to 10³/ml); 1 μCi of "4C-uracil per ml (specific activity, 50 mCi/mmole); "4C-glucose, 0.33 mg/ml; and neopeptone, 0.25 mg/ml. Symbols: total counts; Δ, acid-precipitable counts; O, pool counts (total counts – acid-precipitable counts).

![Figure 5](http://aem.asm.org/)

![Figure 6](http://aem.asm.org/)

![Figure 7](http://aem.asm.org/)

**FIG. 7. Effect of actinomycin D on "4C-uracil incorporation after initiation of germination. Duplicate 100-uliter samples were removed from an incubation mixture containing: macroconidia, 1.7 x 10³/ml; 1 μCi of "4C-uracil per ml (specific activity, 62 mCi/mmole); "4C-glucose, 0.33 mg/ml; and neopeptone, 0.25 mg/ml. a, Initiation of germination, isotope and inhibitor addition, b, germ-tube emergence. Symbols: ●, control; O, 100 μg of actinomycin D per ml.**

**FIG. 6.** "4C-glucose acid-precipitable material accumulated during spore germination*  

| Time (min) | Acid alcohol-soluble counts/min* | Hot acid-soluble counts/min | NaOH-soluble counts/min | Residue counts/min |
|------------|---------------------------------|----------------------------|-------------------------|-------------------|
| 30         | 7,441                           | 357                        | 106                     | 288               |
| 60         | 18,432                          | 978                        | 296                     | 710               |
| 90         | 44,123                          | 2,484                      | 760                     | 1,688             |
| 120        | 93,785                          | 5,834                      | 1,869                   | 8,352             |

*Germination system contained: macroconidia, 10 mg/ml (equivalent to 10³/ml); 1 μCi of "4C-glucose per ml (specific activity, 250 mCi/mmole); "4C-glucose, 0.33 mg/ml; and neopeptone, 0.25 mg/ml.

*Based on 5 mg or 10⁴ macroconidia/ml.
TABLE 3. Effect of increasing concentrations of actinomycin D and cycloheximide upon germ-tube emergence in M. gypseum

| Concentration of inhibitor* (µg/ml) | Spores with germ tubes (% of control) |
|------------------------------------|--------------------------------------|
| Actinomycin D                     |                                      |
| 20                                 | 100                                  |
| 50                                 | 85                                   |
| 100                                | 85                                   |
| Cycloheximide                      |                                      |
| 5                                  | 100                                  |
| 10                                 | 10                                   |
| 50                                 | 0                                    |
| 100                                | 0                                    |

*Inhibitors were added to germination systems containing: macroconidia, 10⁵/ml; ¹⁴C-glucose, 0.33 mg/ml; and neopeptone, 0.25 mg/ml.
*Numbers of germinated macroconidia were determined (after 24 hr) by counting at least 300 macroconidia at each inhibitor concentration.

TABLE 4. Effect of actinomycin D on ¹⁴C-uracil incorporation during germ-tube elongation*

| Actinomycin D (µg/ml) | Min after addition of inhibitor | Counts/min* | % Inhibition | Counts/min* | % Inhibition |
|-----------------------|--------------------------------|-------------|-------------|-------------|-------------|
| 50                    | 30                             | 5.92 x 10⁵  | 0           | 19.05 x 10⁴ | 0           |
| 50                    | 120                            | 4.45 x 10⁴  | 25          | 18.80 x 10⁴ | 2           |
| 25                    |                                | 1.25 x 10⁴  | 78          | 5.02 x 10⁴  | 74          |
| 50                    |                                | 1.14 x 10⁴  | 81          | 5.66 x 10⁴  | 71          |

*Actinomycin D and 1 µCi of uracil-2-¹⁴C per ml, (specific activity, 62 mCi/mmole) were added to germination systems containing: macroconidia, 10⁵/ml; ¹⁴C-glucose, 0.33 mg/ml; and neopeptone, 0.25 mg/ml, after 4 hr (i.e., after germ-tube emergence).
*Counts per minute in cold acid-precipitable material per 10⁴ macroconidia.

is due to permeability changes accompanying germination.

Most of the exogenous glucose taken up was incorporated into acid alcohol-insoluble material (i.e., lipids). One possible explanation for the glucose incorporated into the macroconidial lipid fraction may be a requirement for enhanced membrane synthesis prior to germ-tube emergence and hyphal outgrowth.

The lack of effect of actinomycin D prior to germ-tube emergence may indicate that there is no necessity for the synthesis of new messenger RNA during the earliest stages of germination. Or it may simply reflect a change in permeability of the macroconidium immediately prior to the formation of germ tubes.

TABLE 5. Effect of actinomycin D on ¹⁴C-amino acid incorporation during germ-tube elongation*

| Actinomycin D (µg/ml) | Min after addition of inhibitor | Counts/min* | % Inhibition | Counts/min* | % Inhibition |
|-----------------------|--------------------------------|-------------|-------------|-------------|-------------|
|                      | 30                             |             |             |             |             |
|                      | 90                             |             |             |             |             |
| 0                    |                                | 8.00 x 10⁴  | 0           | 25.00 x 10⁴ | 0           |
| 50                   |                                | 8.28 x 10⁴  | 7           | 4.11 x 10⁴  | 84          |
| 100                  |                                | 8.10 x 10⁵  | 9           | 4.69 x 10⁵  | 82          |

*Actinomycin D and 1 µCi of ¹⁴C-amino acid hydrolyase per ml (specific activity, 63 µCi/ml) were added to germination systems containing: macroconidia, 10⁵/ml; ¹⁴C-glucose, 0.33 mg/ml; and neopeptone, 0.25 mg/ml, after 4 hr (i.e., after germ-tube emergence).
*Counts per minute in cold acid-precipitable material per 10⁴ macroconidia.

Permeability changes associated with germination have been reported for the ascospores of Neurospora tetrasperma (23), and Nishi (16) has suggested that a decrease in phospholipid during the germination of Aspergillus niger conidia might be associated with changes in membrane structure. Griseofulvin also inhibits macromolecular synthesis in M. gypseum only after germ-tube emergence (1). Like actinomycin D,
griseofulvin inhibits germ-tube elongation and not initiation (1), suggesting that a change in permeability might be involved.

On the other hand, there is evidence that the earliest stages in the germination of the zoospores of the aquatic fungus Blastocladiella emersonii (13, 21) and the conidia of the plant pathogens Botryodiplodia theobromae (2) and Peronospora tabacina (7) may utilize performed messenger RNA which is conserved in the ungerminated spore. However, without knowing the effective intracellular concentration of inhibitor at any given time, we should stress that no actinomycin D data can constitute a rigorous demonstration of the existence of a stable messenger RNA. The use of more selective RNA polymerase inhibitors and RNA polymerase mutants should provide a critical test for the presence of such RNA.

Our data would suggest that at the times when actinomycin D is effective, there is a necessity for continuous RNA synthesis for germination to proceed. These results also would suggest caution in the interpretation of any inhibitor studies where there is a possibility of changes in cell permeability.

It would appear that the macroconidia of M. gypseum are prepared to initiate germination and biosynthetic processes immediately upon introduction into a suitable medium. The earliest stages of germination, prior to germ-tube elongation, however, are characterized by a dependence on endogenous substrates (i.e., a low rate of incorporation of labeled metabolites and decreases in dry weight, protein, and RNA values). We would suggest that the macroconidia of M. gypseum are capable of immediate response to a favorable environment and, as such, constitute an ideal means for the transmission of infection. In addition, it would appear that a number of products necessary for germination may be synthesized late in the sporulation cycle (10-12, 17, 18).

The decreases in specific activity reported for several metabolic enzymes (12) and the apparent turnover of macromolecular constituents documented here are consistent with our previous suggestion (12) that the longevity of the macroconidium may depend more on a high concentration of essential materials in the spore rather than any well developed dormancy mechanisms. Since macroconidia are known to have little increased thermal resistance as compared to vegetative mycelia (23), it would appear that their primary importance may be as an easily disseminated means for the rapid establishment of new growth in a suitable environment.

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