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# Characterization of L-Leucine-Induced Germination of *Trichophyton mentagrophytes* Microconidia

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L-Leucine and several amino acids were effective germination inducers of microconidia of *Trichophyton mentagrophytes*. During germination, phase-darkening and swelling occurred concomitantly with the loss of resistance to heat and stain, reduction of dry weight and specific gravity, and development of active glucose utilization. Germination induced by L-leucine was significantly stimulated by a pretreatment of the spores with sublethal doses of heat. No nucleosides or nucleotides were stimulatory to the L-leucine-induced germination of the microconidia. D-Leucine was almost an equally effective germinant as its L form. No carbohydrates, salts, vitamins, or other compounds tested induced germination of the fungus spore. Other factors that affected L-leucine-induced germination of the microconidia included the concentration of leucine, the inoculum size of the spores, temperature, and *p*H. The anaerobic condition and the presence or absence of carbon dioxide had no significant effects on the germination. Short germ tubes usually developed when the germinated spores were further incubated either in the presence or absence of L-leucine. The cytological study of the germinating microconidia revealed that the fragmentation of lipid granules was the major structural change associated with the germination.

Fungal spores are considered germinated when the protrusion of the germ tube from the spore wall becomes microscopically evident (7, 14). Although this criterion of germination is widely accepted in routine work in fungi, there appears to be no general agreement among mycologists as to the exact point at which fungal spores are considered germinated (7). Because some morphological and physiological changes have been known to occur in fungal spores prior to the germ tube emergence (4, 12-14, 18), it is possible that dormant fungal spores undergo germination through a few well-defined stages.

During the course of our search for factor(s) initiating germination of microconidia of *Trichophyton mentagrophytes*, we found that the dormant spores, which appear refractile under a phase-contrast microscope, become phase-dark prior to germ tube development in the presence of L-leucine or several other amino acids and that during such change they lose several other properties characteristic of dormant spores (Hashimoto, Wu, and Blumenthal, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 56, 1972). The present paper describes the morphological and physiological changes associated with the initial stage of germination of *T. mentagrophytes* microconidia induced by L-leucine and some factors affecting such germination. On the basis of data presented, we suggest subdivision of the germination process of fungal spores into two distinct stages, initiation and germ tube emergence.

## MATERIALS AND METHODS

**Organism.** *T. mentagrophytes* strain SF306A/68, obtained from J. Rippon, University of Chicago, was used throughout this investigation. The fungus was maintained on slants of the modified Sabouraud glucose agar (Difco, Detroit, Mich.) containing 20 g of glucose, 10 g of peptone, and 15 g of agar in 1 liter of medium.

**Preparation and purification of microconidia.** Abundant microconidia were formed by this fungus when grown on Sabouraud agar medium containing 4% glucose at 30 C for 1 to 2 weeks. The microconidia were dislodged from the agar surface by gentle

scraping with a sterile glass rod covered with a Tygon tube. The mass of spores from one petri plate were then transferred into a test tube containing approximately 2 ml of sterile distilled water and gradually were made into a homogeneous suspension by gently rubbing the spores with the Tygon tube-coated glass rod against the test tube wall. Filtration of the spore suspension through about 10 layers of sterile cheesecloth proved useful in eliminating residual mycelia and undispersed spore masses. These spores were repeatedly washed, usually five to eight times, with sterile, distilled water until microscopically clean spore suspensions were obtained. To avoid the initiation of germination of these spores during harvesting and washing, the whole purification procedure was carried out in a cold room (4 C). Microconidia preparations showing less than 10% of nonrefractile spores after incubation at 37 C for 24 hr in a sodium phosphate buffer (*p*H 6.0, 0.1 M) were considered clean and were used in our experiments. The washed spores were dispensed in small vials and kept frozen at −10 C until use.

**Phase-contrast photomicroscopy.** The microscopic appearance of the wet-mounted spores was examined with a phase-contrast microscope by using an oil immersion objective (dark medium, ×100; numerical aperture, 1.25; Nikon). Photomicroscopy was made on panchromatic film (Kodak Plus-X) with a Nikon camera equipped with an automatic exposure system attached to a phase-contrast microscope.

**Lipid stain.** Lipid granules of spores were stained by the method of Burdon (2) by using 0.3% Sudan black B.

**Germination system.** Unless otherwise stated, 22 by 75 mm test tubes containing 1 ml of *T. mentagrophytes* microconidial suspension ($5 \times 10^6$ spores/ml) were incubated in the presence of an appropriate concentration of a germinating agent on a rotary shaker (model G-10, New Brunswick Scientific Co., N.J.) at 200 rev/min under specified conditions. Samples were removed at appropriate intervals, and percentages of nonrefractile spores were estimated microscopically by counting a total of 200 microspores.

In the present study, microconidia were considered initiated when the loss of refractility was observed under a phase-contrast microscope and were counted as germinated spores. The effects of various chemicals on L-leucine-induced germination were tested by incorporating a specified concentration of a test compound into the germination system described above.

**Heat activation.** To test the effect of sublethal heat treatment on the L-leucine-induced germination, spores were pretreated at 40 and 45 C for varying lengths of time before being mixed with L-leucine. Our preliminary tests showed that about 10% of spores were killed when they were heated at 50 C for 30 min.

**Heat resistance.** Dormant spore suspensions were heated at 55 C for various periods of time and chilled in an ice bath; then 0.1 ml of appropriate dilutions were plated on Sabouraud glucose agar. After 3 days of incubation at 30 C, the colonies were counted and the percentage of survival was computed. The heat resistance of germinated spores was determined by a microscopy method (9). In this study, a phase-contrast microscope was used in counting elongated cells without staining. Use of this method for germinated spores was necessitated because of some clumping during germination.

**Stainability with basic dyes.** A few drops of 0.5% crystal violet or methylene blue solution were placed upon a glass slide smeared with a thin film of spores and covered with a thin cover glass (no. 1 thickness). The spores which failed to stain within 10 min at room temperature were regarded as resistant to the stain.

**Determination of the size and dry weight of spores.** Spore sizes were determined by an Electrozone Celloscope (Particle Data, Inc., Elmhurst, Ill.) by counting more than 25,000 cells for each sample. The dry weight of spores suspended in water was determined after drying in an oven at 90 C for 48 hr.

**Separation of dormant and germinated microconidia by using gradient centrifugation.** The reduction in specific gravity of microconidia after initiation of germination was demonstrated by means of gradient centrifugation by using Renografin (Reno M-60, E. R. Squibb & Sons, Inc., New York). The method used in preparing linear gradients was essentially the same as that described by Tamir and Gilvarg (15). The tubes were centrifuged in an International Equipment Co. centrifuge (model B35) equipped with a swinging bucket rotor (no. SB 269) at 17,000 rev/min for 30 min. Our preliminary tests showed that exposure to Renografin has no detrimental effects on either dormant or germinated spores of *T. mentagrophytes*.

**Oxygen uptake studies.** Both dormant and germinated microconidia, at a final concentration of $7 \times 10^6$ cells per ml in sodium phosphate buffer (0.1 M, *p*H 7.0), were used. Oxygen uptake by the cells in the presence of glucose (50 mM) was measured manometrically at 37 C by standard methods (16).

**Chemicals.** All of the amino acids and dipeptides used were chromatographically pure and were purchased from Sigma Chemical Co., St. Louis, Mo. Unless specifically mentioned otherwise, all amino acids and dipeptides refer to the L isomers. The purines and pyrimidines also were purchased from Sigma Chemical Co., whereas carbohydrates and other biochemicals were from Nutritional Biochemical Co., Cleveland, Ohio. All other chemicals were of reagent grade.

## RESULTS

The dormant microconidia of *T. mentagrophytes* lost their refractility when incubated in 0.5% peptone broth, or in Sabouraud broth containing 2% glucose (Fig. 1a, b, and c). In the presence of L-leucine only (25 mM), the process ceased at the stage represented in Fig. 1d, and a supplementation of additional nutrients was essential for the subsequent germ

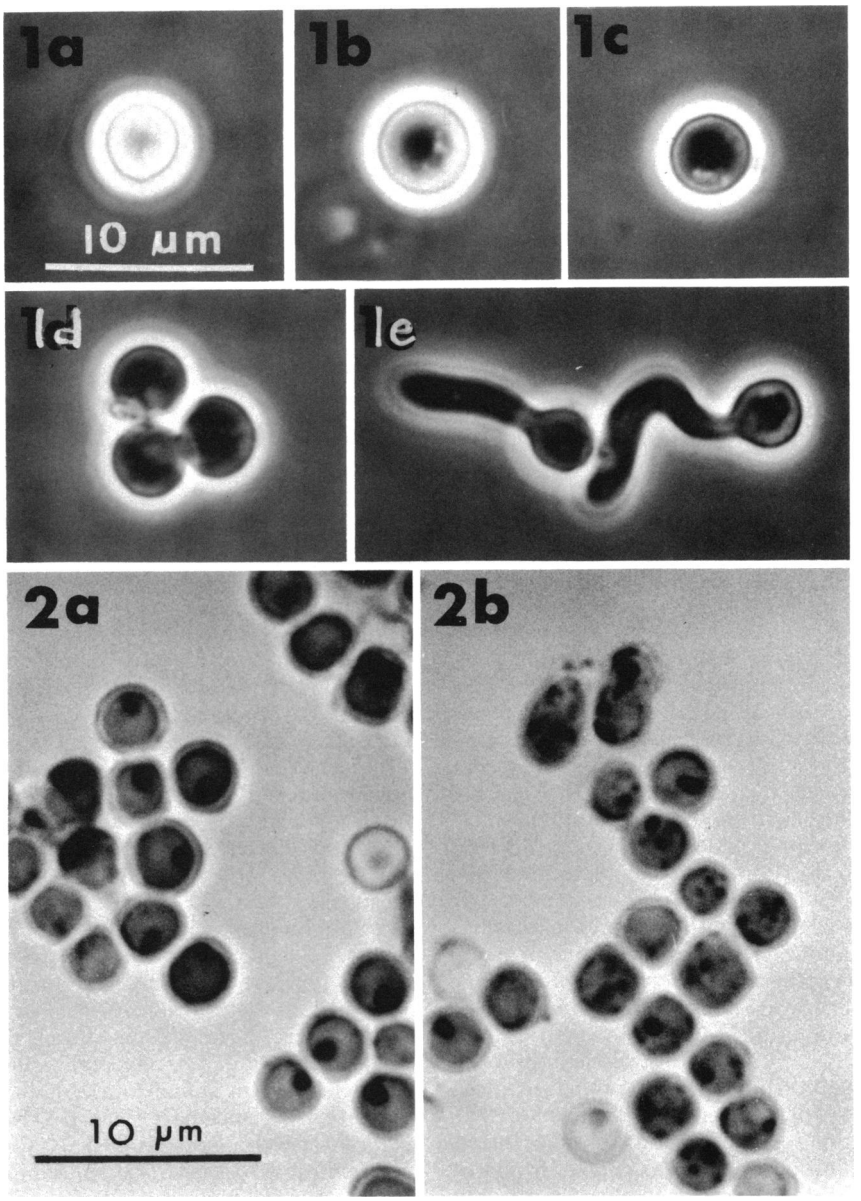


FIG. 1. *Phase-contrast photomicrographs showing the transformation of dormant T. mentagrophytes microconidia into vegetative hyphae in 0.5% peptone broth. Note that the refractility of dormant microconidia (1a) is gradually lost (1b and 1c), and eventually germ tubes emerge (1d and 1e). The photomicrographs were taken 0 hr (1a), 1 hr (1b), 2 hr (1c), 3 hr (1d) and 6 hr (1e) after inoculation and represent the appearance of microconidia most predominant in the population at the specified time.*

FIG. 2. *Light micrographs of T. mentagrophytes microconidia stained with Sudan black B. Note the changes in profile of lipid granules before (2a) and after (2b) initiation of germination.*

tube elongation (Fig. 1e). Usually, the transition from dormant (Fig. 1a) to phase-dark spores (Fig. 1c) takes approximately 2 hr at 37 C and an additional hour is necessary to reach the stage represented by Fig. 1d. The loss of refractility also occurred when the complex media were replaced by the chemically defined Wickerham yeast-nitrogen base (without amino acids; Difco, Detroit, Mich.) individually supplemented with certain of the 22 L-amino acids and seven dipeptides listed in Table 1. The subsequent search for the effec-

TABLE 1. *Germination of T. mentagrophytes microconidia induced by L-amino acids and dipeptides*

| Compound | Activity[a] | Compound | Activity[a] |
|---|---|---|---|
| Amino acids | | | |
| Alanine | 30–60 | Serine | <10 |
| Arginine hydrochloride | <10 | Threonine | <10 |
| Asparagine | <10 | Tryptophan | 30–60 |
| Aspartic acid | <10 | Tyrosine[b] | <10 |
| Cysteine | <10 | Valine | 30–60 |
| Cystine[b] | <10 | Glycine | 30–60 |
| Glutamine | <10 | Dipeptides | |
| Glutamic acid | <10 | Leucyl-leucine | 30–60 |
| Histidine hydrochloride | <10 | Leucyl-tyrosine | 30–60 |
| Hydroxy-L-proline | <10 | Leucyl-valine | 30–60 |
| Isoleucine | 30–60 | Valyl-valine | 10–30 |
| Leucine | 60–95 | Valyl-tyrosine | 10–30 |
| Lysine hydrochloride | <10 | *N*-Carbamyl-leucine | <10 |
| Methionine | 30–60 | Leucine ethyl ester hydrochloride | 10–30 |
| Phenylalanine | 10–30 | Phosphate buffer only | |
| Proline | 10–30 | (0.1 M, *p*H 6.0) | <10 |

[a] Percent of germinated spores after incubation at 37 C for 8 hr in the presence of 25 mM of each compound in sodium phosphate buffer (0.1 M, *p*H 6.0) as described in Materials and Methods.

[b] Saturated solutions were used due to the limited solubility of these compounds.

tive factor(s) which triggers the germination of *T. mentagrophytes* microconidia resulted in the identification of seven amino acids: leucine, isoleucine, valine, alanine, tryptophan, methionine, and glycine. Of these, L-leucine was found to be the most effective germinating agent for the *T. mentagrophytes* microconidia (Table 1). Although the rate of germination by leucine (25 mM) is slower than that in 0.5% peptone broth, the almost complete germination, as judged by phase-contrast microscopy, was always achieved within several hours (Fig. 3). No germination took place either in phosphate buffer, other buffer solutions, or in water (Fig. 3). Those microconidia germinated by leucine or any other single amino acid usually formed short germ tubes (up to one-half of the diameter of the spore) after a prolonged incubation (Fig. 1d). It is interesting to note that the formation of short germ tubes occurred even if the initiated spores, as judged by phase-contrast microscopy, were washed thoroughly in phosphate buffer (0.1 M, *p*H 6.0), suspended in the buffer, and incubated in the absence of leucine. The supplementation of additional nutrients was essential for the further elongation of the germ tubes (Fig. 13). The exact nutritional requirements for the vegetative growth remain to be determined.

**Germination inducers other than leucine.** A number of nitrogenous compounds, organic acids, and carbohydrates were tested for their ability to induce germination of *T. mentagrophytes* microconidia. No compounds other than certain amino acids and dipeptides (Table 1) were capable of causing germination of the microspores to any significant extent. The following carbohydrates, organic acids, purines, pyrimidines, nucleosides, and other nitrogenous compounds failed either to induce germination by themselves or to stimulate L-leucine-induced germination of the microconidia.

**Carbohydrates and organic acids.** Carbohydrates and organic acids (0.5% in sodium phosphate buffer, 0.1 M, *p*H 6.0) were as follows: D-glucose, D-mannose, L-mannose, D-fructose, D-galactose, D-ribose, L-arabinose, D-xylose, L-xylose, D-rhamnose, D-turanose, D-cellobiose, D-melezitose, D-trehalose, D-melibiose, sucrose, L-sorbose, D-sorbitol, L-arabitol, *iso*-erythritol, dulcitol, glycerol, adonitol, arabic acid, inulin, salicin, amygdalin, dextrin, pyruvate (Na), succinate (Na), α-ketoglutarate (Na), *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, *N*-acetyl-D-mannosamine, and D-galactosamine hydrochloride.

**Purines, pyrimidines, and nucleosides.** Purines, pyrimidines, and nucleosides (20 mM in 0.1 M sodium phosphate buffer, *p*H 6.0) were as follows: adenine, cytidine sulfate, guanine, uracil, xanthine, hypoxanthine, adenosine, cytosine, guanosine, uridine, 5-methylcytosine-hydrochloride, and inosine.

**Miscellaneous compounds.** Miscellaneous compounds (0.1% aqueous solution) were as follows: urea, ammonium nitrate, ammonium sulfate, and potassium nitrate.

The survey also revealed that some dipeptides containing L-leucine were reasonably

good germination inducers for the microconidia (Table 1). Similarly, the D isomer of L-leucine was found to induce the germination of the spores, although somewhat less efficiently when compared with its L isomer (Table 2).

Although the rate of germination of the dormant microconidia mediated by any single amino acid never reached that of a complex medium (0.5% peptone, for instance), the germination of the microconidia in the presence of all seven amino acids (25 mM each) was almost as rapid as in the complex medium. Supplementation with various carbohydrates failed to stimulate the amino acid-induced germination of the microconidia. High concentrations of glucose retarded somewhat the leucine-induced germination of *T. mentagrophytes* microconidia (Table 3).

**Effects of vitamins and minerals.** No vitamins or minerals contained in the Wickerham yeast-nitrogen base (17) appear to be required for the initiation or the completion of germination of the microconidia induced by leucine. The germination of the microconidia induced by leucine was only slightly affected, if at all, by buffer concentrations in the range tested (0.01 to 0.2 M).

**Cytological and physical changes associated with germination.** In addition to the loss of refractility, several other physical and cytological changes occurred in the microconidium during germination.

The analysis of the cell size by an Electrozone Celloscope showed an increase in diameter (approximately 10%) after initiation of germination (Table 4). The data in Table 4 and Fig. 4 show that there was a concomitant reduction in the dry weight and in the specific gravity of the microconidia as they germinated.

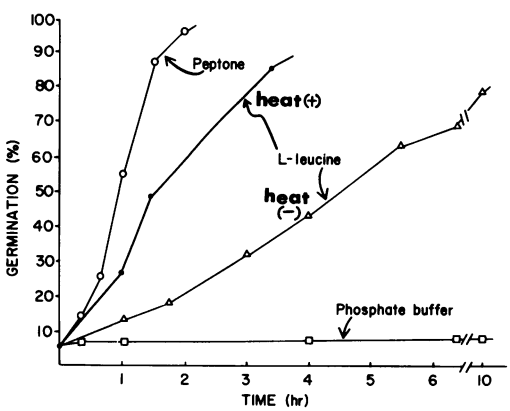


FIG. 3. *Germination curves of T. mentagrophytes microconidia (5 × 10⁶ spores/ml) in the presence of 0.5% peptone broth or L-leucine (25 mM) or in sodium phosphate buffer (0.1 M, pH 6.0). The activation effect of a sublethal heat treatment (45 C for 30 min) on leucine-induced germination of T. mentagrophytes microconidia is illustrated. The incubation temperature was 37 C in all experiments.*

TABLE 2. *Effect of L- and D-leucine concentration on the germination of heat-activated microconidia of T. mentagrophytes*

| Concn (mM) | Germination (%)[a] | | | | | |
|---|---|---|---|---|---|---|
| | L-Leucine | | | D-Leucine | | |
| | 4 hr | 8 hr | 18 hr | 4 hr | 8 hr | 18 hr |
| 25 | 82 | 95 | 98 | 65 | 84 | 96 |
| 10 | 68 | 91 | 96 | 74 | 87 | 96 |
| 1 | 64 | 87 | 95 | 41 | 58 | 92 |
| 0.1 | 54 | 68 | 96 | 21 | 26 | 88 |

[a] Percent of germinated spores after incubation at 37 C for 4, 8, and 18 hr, respectively, as described in Materials and Methods. Less than 10% of the microconidia germinated in the absence of L-leucine. The germination system contained 5 × 10⁶ spores in 1 ml of phosphate buffer (0.1 M, pH 6.0) containing the specified concentration of the germinating agent.

TABLE 3. *Effect of glucose on heat-shocked (45 C, 30 min) T. mentagrophytes microconidia germination induced by leucine*

| Glucose concn (%) in presence of L-leucine (25 mM) | Germination (%)[a] | |
|---|---|---|
| | 1.5 hr | 3 hr |
| 4 | 17 | 32 |
| 2 | 20 | 50 |
| 1 | 28 | 65 |
| 0.5 | 33 | 47 |
| 0 | 35 | 64 |
| Phosphate buffer control (0.1 M, pH 6.0) | <10 | <10 |

[a] Percent of germination after 1.5 and 3 hr of incubation at 37 C. The concentration of spores in all experiments was 5 × 10⁶/ml.

Dormant, unfixed spores were difficult to stain at room temperature with common basic dyes such as methylene blue or crystal violet at concentrations ranging from 0.1 to 0.5%, whereas the germinated cells were readily stained with the same dye solutions. Another cytological change observed in the germinated spores was the size of lipid granules. As illustrated in Fig. 2a and b, the dormant spores contained large lipid granules which apparently underwent fragmentation during germination. The actual changes in lipid content were not determined.

TABLE 4. *Dry weight, average cell size, and the glucose oxidizing activity of dormant and germinated T. mentagrophytes microconidia*

| Conditions of spores | Dry wt $1.5 \times 10^8$ spores (mg) | Cell diameter | | Glucose oxidation[a] (counts/min) | |
|---|---|---|---|---|---|
| | | Total no. counted | Mean ($\mu$m) | Glucose-*1*-$^{14}C$ | Glucose-*6*-$^{14}C$ |
| Dormant | 4.06 | 27,691 | 3.52 ± 0.26 | 765 | 649 |
| Germinated[b] | 3.71 | 30,735 | 3.88 ± 0.18 | 4,826 | 1,569 |

[a] Measured as $^{14}CO_2$ evolved from glucose-*1*-$^{14}C$ or -*6*-$^{14}C$ during 4 hr. Each reaction flask contained 2.5 ml of spore suspension ($3 \times 10^7$ cells/ml), 1 ml of sodium phosphate buffer (0.1 M, *p*H 6.0), and 0.5 ml of $^{14}C$-labeled glucose solution (25 $\mu$moles) containing a total of approximately 50,000 counts/min and incubated at 37 C. The detailed account of the isotope experiments were previously described by Blumenthal (1).

[b] Obtained by incubating dormant spores in 0.5% peptone for 2 hr at 37 C, which permitted a more synchronous germination than any other system used. Essentially no spores have germ tubes at this stage. Aggregated cells were removed by light centrifugation and only well-dispersed single-spore suspensions were used in the experiments.

**Changes in physiological properties of microconidia occurring during germination.** The microconidia lost their heat resistance during germination. The representative thermal inactivation curves of both dormant and germinated spores exposed to 55 C are illustrated in Fig. 5. In the dormant spores, the loss of both viability and germinability (ability to respond to leucine to become phase dark) occurred almost simultaneously when spores were exposed to lethal doses of heat. Those spores lethally treated with heat were unable to germinate either in Sabouraud broth or agar medium (data not shown). In contrast, there appeared to be no significant difference in resistance to ultraviolet light (UV) between dormant and germinated spores (Fig. 6). Those spores apparently killed by UV irradiation were still capable of initiating germination in the presence of leucine (Fig. 6), but no discernible germ tube formation was observed.

There was also a notable difference in respiratory activity between dormant and germinated spores. As shown in Fig. 7, the dormant microconidia did not respire in the presence of D-glucose, whereas the germinated spores showed a significant oxygen uptake after a lag of 30 min (Fig. 7). That this increased rate of oxygen uptake was due largely to active glucose use by germinated microspores rather than to a stimulation of the use of endogenous materials was confirmed by the experiments with $^{14}C$-labeled D-glucose (Table 4). The oxidation of $^{14}C$-glucose to $^{14}CO_2$ was significantly stimulated in the germinated microconidia.

**Factors affecting leucine-induced germination of T. mentagrophytes microconidia: effects of pH and temperature.** Although the germination of the microconidia occurred over a wide range of *p*H, the optimal *p*H appeared to be between 6.0 and 6.5 (Fig. 8). The microconidial germination was found to be greatly influenced by incubation temperature. The germination occurred best at 37 C. The optimal temperature for the vegetative growth of this fungus was 30 C (Fig. 9) and the optimal temperature for sporulation was 25 C.

**Activation by heat.** Pretreatment with sublethal doses of heat had an appreciable effect on the rate of the leucine-induced germination of the microconidia (Fig. 3). The microconidia

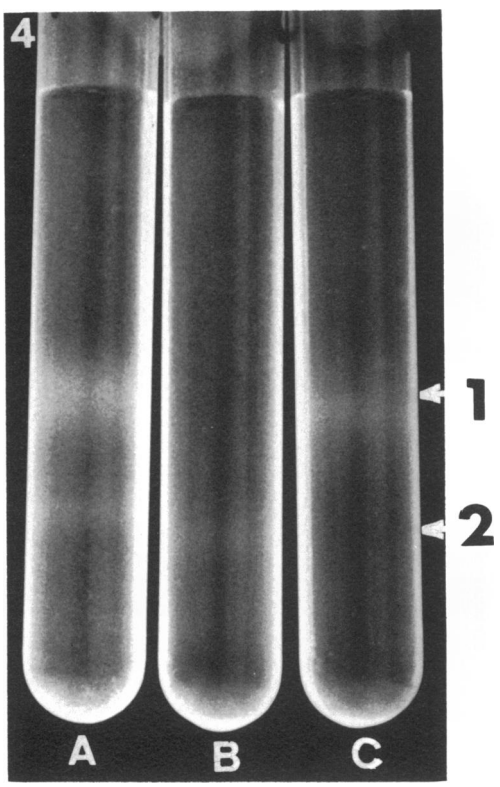


FIG. 4. *The Renografin density gradient tubes showing the separation bands of germinated (arrow 1) and dormant (arrow 2) T. mentagrophytes microconidia. Tube A contains the mixture of germinated and dormant microconidia, tube B contains dormant microconidia only, and tube C contains germinated microconidia only. The gradient range of Renografin is 40% at the top and 80% at the bottom.*

exposed to 40 and 45 C for varying periods of time (up to 30 min) prior to the addition of L-leucine or peptone broth (0.5%) germinated more rapidly than those which received no heat treatment. A significant increase of germination rate after a heat treatment (30 min at 45 C) is illustrated in Fig. 3.

**Effects of leucine concentration and inoculum size of microconidia.** When a moderate concentration of microconidia ($5 \times 10^6$ spores/ml) was used, the concentration of leucine affected the rate, but not the extent, of germination within the range of leucine concentration tested (Table 2). Almost complete germination of microconidia was achieved within 18 hr at a leucine concentration as low as 0.1 mM when $5 \times 10^6$ spores/ml were used (Table 2). However, at a given concentration of leucine (25 mM) the extent of germination appeared to bear a negative relationship to inoculum size of spores (Table 5).

**Effects of anaerobic condition and carbon dioxide.** Although *T. mentagrophytes* requires aerobic condition for vegetative growth, rapid microconidial germination could be obtained in the almost complete absence of oxygen (100% $N_2$) or even in vacuum (data not shown). Carbon dioxide (95% $CO_2$ and 5% $N_2$ or 0% $CO_2$, 80% $N_2$, and 20% $O_2$) had no appreciable

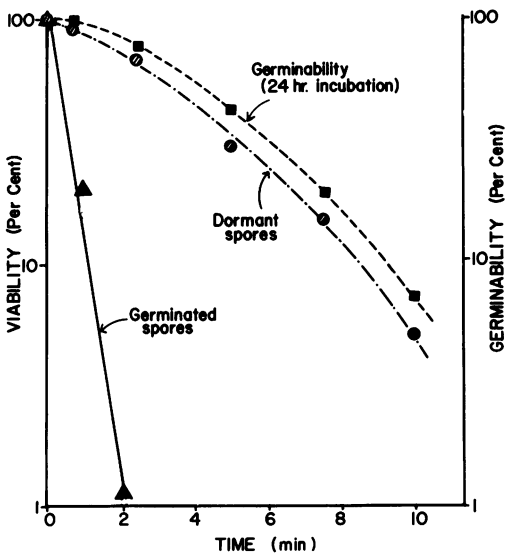


FIG. 5. *Heat inactivation curves of dormant and germinated T. mentagrophytes microconidia illustrating the loss of heat resistance during germination. This also illustrates that dormant microconidia exposed to lethal doses of heat were unable to become phase dark in the presence of L-leucine, contrasting the preservation of such ability in UV-killed spores (see Fig. 6). The spores were treated at 55 C for specified periods and the viability of the heated spores were determined as described in Materials and Methods. The loss of germinability on leucine was determined microscopically after incubation of spores for 24 hr at 37 C in the presence of L-leucine (25 mM in phosphate buffer, 0.1 M, pH 6.0).*

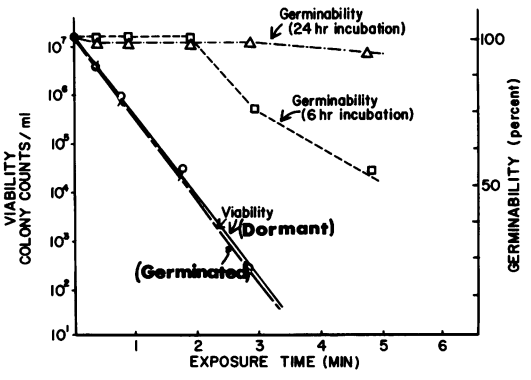


FIG. 6. *Ultraviolet light inactivation curves of dormant and germinated T. mentagrophytes microconidia. A 2-ml amount of a spore suspension ($1 \times 10^7$ spores/ml) was placed in a small petri dish (40 × 10 mm) and irradiated at room temperature for specified periods of time at a distance of 42 cm from the center of a germicidal lamp (Westinghouse Sterilamp 782L-20). Note that the germinability on L-leucine (25 mM in phosphate buffer, 0.1 M, pH 6.0 at 37 C) is maintained long after the viability has been lost. There was no significant difference in UV resistance between the dormant and germinated microconidia.*

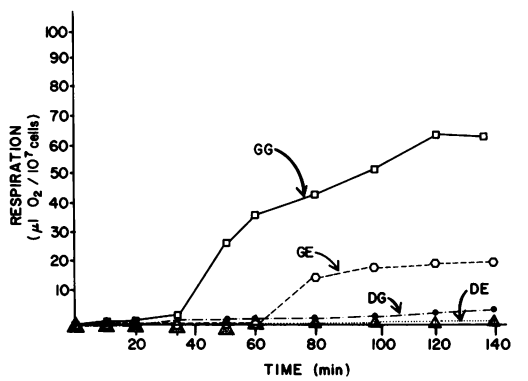


FIG. 7. *Oxygen uptake of dormant and germinated microconidia in the presence or absence of glucose as determined by manometric techniques. Germinated spores respire either in the presence (GG) or absence (GE) of glucose. No significant oxygen uptake was found in the dormant spores regardless of the presence (DG) or absence (DE) of glucose. The reaction flasks contained 1 ml of spore suspensions ($2.6 \times 10^7$ cells/ml) and 1 ml of 100 mM D-glucose solution in sodium phosphate buffer (0.1 M, pH 7.0).*

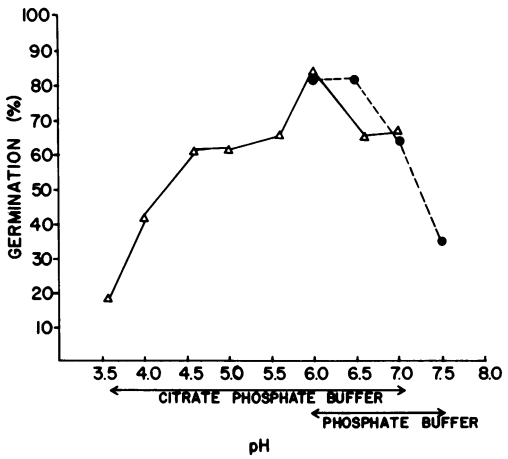


FIG. 8. *Effects of pH on the leucine induced germination of T. mentagrophytes microconidia. The optimal pH appears to be at 6.0 to 6.5, although fair degrees of germination can take place over a wide range of pH (4.0 to 7.5). The germination system contained 5 × 10⁶ spores/ml of 25 mM leucine solution in a buffer and was incubated at 37 C for 8 hr. The concentration of the buffer was 0.1 M. ●, Phosphate buffer; △, citrate phosphate buffer.*

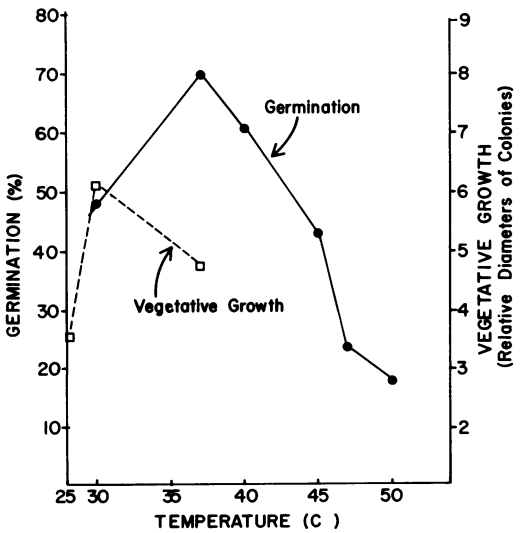


FIG. 9. *Effects of temperature on leucine-induced germination of microconidia and on vegetative growth of T. mentagrophytes. The germination system contained 5 × 10⁶ spores/ml of 25 mM leucine solution in a sodium phosphate buffer (0.1 M, pH 6.0) and incubated for 8 hr at temperatures specified. The vegetative growth was determined by measuring the diameter of colonies growing on the Sabouraud agar medium containing 4% glucose at the specified temperatures after 2 weeks.*

TABLE 5. *Effect of spore concentration on leucine-induced germination of T. mentagrophytes microconidia*

| No. of spores/ml (× 10⁶) | Germination (%)[a] | | | | |
|---|---|---|---|---|---|
| | 3 hr | 6 hr | 9 hr | 18 hr | 24 hr |
| 15.2 | 30 | 48 | 48 | 50 | 52 |
| 10.8 | 40 | 53 | 54 | 54 | 54 |
| 8.2 | 35 | 48 | 67 | 74 | 75 |
| 6.2 | 49 | 73 | 78 | 86 | 88 |
| 4.5 | 38 | 72 | 74 | 98 | 98 |
| 3.4 | 42 | 79 | 89 | 98 | 98 |

[a] Percent of germinated spores after incubation at 37 C in the presence of 25 mM L-leucine in sodium phosphate buffer (0.1 M, pH 6.0) for specified periods of time.

effects on the germination of the microconidia (data not shown).

## DISCUSSION

To our knowledge, no single compound which triggers the germination of *T. mentagrophytes* microconidia has been previously identified. The present study has clearly demonstrated that *T. mentagrophytes* microconidia undergo rapid phase darkening and swelling in the presence of L-leucine only prior to the development of germ tubes and that the germ tube protrusion from initiated spores may occur in the absence of exogenous nutrients once the dormancy is broken. These findings have provided an excellent experimental system whereby one can pursue the changes associated with different stages of the transformation of the dormant fungus spores to vegetative cells under defined conditions.

The ability of the initiated (phase-dark) microconidia to develop short germ tubes in the phosphate buffer is attributed to the presence of endogenous materials, most likely abundant lipids, although the use of a trace amount of leucine remaining after washing cannot be totally excluded. The breakage of dormancy is apparently the prerequisite for the use of the endogenous materials. Although germination can be initiated in bacterial spores by simple compounds such as amino acids or sugars, a supply of exogenous nitrogen and carbon sources is essential for the postgerminative elongation and outgrowth of germinated bacterial spores (14). In fungal spores, on the other hand, once the dormancy is broken by a triggering agent, the amount of endogenous materials available in fungus spores for the subsequent vegetative growth seems to be

the limiting factor in determining the extent of germ tube elongation. The presence or absence of such endogenous materials appears to be the major difference between fungus and bacterial spores in the postgerminative development system. The independent nature of reaction(s) involved in breaking dormancy from those of the germ tube protrusion is further evidenced by the observation that the UV-killed microconidia retain full germinability while being deprived of their ability to form germ tubes. In the case of *T. mentagrophytes* microconidia, germination should be considered as initiated when they have turned phase dark and lost resistance to heat and dyes. Recently, loss of refractility (10) and acid fastness (11) was reported to occur in association with germination of ascospores of *Saccharomyces cerevisiae*. Loss of heat resistance (6), dry weight (4, 10), and lipid (3) was observed during germination of other fungus spores when germination was defined by germ tube formation. However, the findings in the present study represent the first fungal spore system in which the major consequences of germination in bacterial spores are also found in fungal spores. These consequences are phase darkening, loss of heat resistance and resistance to staining, increase in size, and decrease in dry weight and density.

The inability of UV-killed microconidia to form germ tubes, while retaining the ability to become phase dark by L-leucine, is interpreted as meaning that the enzymes necessary for the use of the endogenous substance for germ tube development may have to be synthesized de novo, whereas the enzymes involved in breaking dormancy are stored preformed. The marked inhibition of the process leading to phase darkening of the microconidia by phenylmethylsulfonyl fluoride (*unpublished data*), a known inhibitor of protease, may imply the involvement of proteases or other hydrolytic enzymes in such reactions. The possible involvement of lysosome enzymes in germination of macroconidia of *Microsporum gypseum* has been reported recently (8). An inhibition of microconidial germination by a high concentration of D-glucose (Table 3) may be due in part to the interference by this sugar with the enzymatic degradation of spore wall glucan (*unpublished data*).

The degradative nature of the initial phase of germination is inferred from the decrease of both the dry weight and the specific gravity of germinated spores as compared with their dormant counterpart (Fig. 4 and Table 4). Whether such changes were due to the release into the surrounding medium of certain spore components required for maintaining dormancy or to the progressive degradation of spore materials in the absence of the synthesis of new cell materials is still unknown.

The imbibition of water and the enzymatic weakening of the spore wall may be jointly responsible for the slight increase of size of microconidia (Table 4) during germination. Swelling is known to occur in many fungal spores during germination (4, 7). The assumption that the poor stainability of dormant spores with basic dyes is due to their limited permeability was verified by the observation that the dormant spore cytoplasm stained intensely with basic dyes when the wall was slightly damaged by crushing between slide and cover glasses. The loss in optical density of spore suspensions has been reported to occur during germination of bacterial spores (14). However, the use of this parameter for following the course of germination of *T. mentagrophytes* microconidia was found unsuitable because of clumping during germination.

The demonstration of metabolic activities, as evidenced by active glucose utilization by phase-dark microconidia (Table 4), is another indication of the loss of dormancy at this stage. A lag preceding the initiation of oxygen uptake by initiated *T. mentagrophytes* microconidia (Fig. 7) may indicate that some enzymes essential for glucose utilization are induced during this period. Activated or germinated fungal spores were reported to respire actively in the presence of glucose (13, 14).

The results of studies dealing with the environmental factors affecting the leucine-induced germination strongly suggest that the initiation reaction(s) is enzymatic, although the exact nature of such a reaction(s), or the role of L-leucine in the triggering mechanism, is not known at this time. As reported in bacterial spores (5, 14) and some other fungal spores (12, 13), the germination of microconidia is greatly stimulated by pretreatment of the dormant spores with sublethal doses of heat (Fig. 3). So far, no compounds have been shown to enhance the leucine-induced germination of this fungal spore. Although D-alanine is reported to competitively inhibit the germination of bacterial spores induced by L-alanine (14), D-leucine did not interfere with the L-leucine-induced germination of *T. mentagrophytes* microconidia. In fact, D-leucine itself was a good germinant, although not as good as L-leucine (Table 2). To our knowledge, no leu-

cine racemase has been reported in *T. mentagrophytes* spores. The observations that *T. mentagrophytes* microconidia could germinate under anaerobic conditions or in the absence of carbon dioxide are of interest in view of the obligately aerobic nature of this fungus and the reported $CO_2$ requirement for germination of certain conidia (18) and bacterial endospores (14). It is relevant to recall that the endospores of aerobic bacilli can germinate either in the presence or absence of oxygen provided an appropriate germinant is present (14).

It is hoped that additional studies of the germination of this fungus spore, under defined conditions, may provide clues to the elucidation of the mechanism involved in the breaking of dormancy of the microconidia of this fungus and of other fungal spores.

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