1973

Germination of Trichophyton Mentagrophytes Microconidia

Christine Da-Ruh Wu

Loyola University Chicago

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GERMINATION OF
TRICHOPHYTON MENTAGROPHYTES MICROCONIDIA

by
Christine Da-Ruh Wu

A THESIS SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF LOYOLA UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE

FEBRUARY
1973
DEDICATION

TO MY PARENTS, IN TESTIMONY OF MY GRATITUDE FOR
THEIR LOVE, GUIDANCE AND SACRIFICE.
ACKNOWLEDGEMENTS

My deepest appreciation is expressed to Dr. Tadayo Hashimoto for his encouragement, patience and, most of all, his endless hours of counsel and effort rendered throughout the entire study.

To Dr. H. J. Blumenthal, Chairman of the Department, goes my heartfelt gratitude for his kind advice and assistance.

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Finally, I am indebted to Mrs. Eileen Hebenstreit for her patience and skillfulness in typing this thesis.
Life

Christine Da-Ruh Wu was born to Mr. and Mrs. D. K. Wu in Shanghai, China on December 5, 1948. She graduated from the Provincial First Girls' High School, Taipei, Taiwan in June, 1966 and received a Bachelor of Science degree from the Department of Botany, College of Science, National Taiwan University, Taipei, Taiwan in June, 1970. In September of the same year, she began her studies for a Master's degree in the Department of Microbiology, the Loyola University of Chicago, Stritch School of Medicine. Miss Wu was the first place winner in the Graduate Research Forum for student papers sponsored by the Loyola University Chapter of Sigma Xi in May of 1972.
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<tr>
<td>C</td>
<td>centigrade</td>
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<td>cm</td>
<td>centimeter</td>
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<td>concn</td>
<td>concentration</td>
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<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
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<td>g</td>
<td>gram</td>
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<td>hr</td>
<td>hour</td>
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<tr>
<td>kV</td>
<td>kilovolts</td>
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<tr>
<td>KU</td>
<td>Klett unit</td>
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<td>l</td>
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<td>leu</td>
<td>leucine</td>
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<td>M</td>
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<td>nm</td>
<td>nanometer</td>
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<td>%</td>
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<tr>
<td>PMSF</td>
<td>phenylmethysulfonyl fluoride</td>
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<tr>
<td>rev</td>
<td>revolution</td>
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<tr>
<td>SGA</td>
<td>Sabouraud glucose agar</td>
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<td>SGB</td>
<td>Sabouraud glucose broth</td>
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<td>sec</td>
<td>second</td>
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<tr>
<td>UV</td>
<td>ultra violet</td>
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<tr>
<td>w/v</td>
<td>weight per volume</td>
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<td>YNB</td>
<td>Wickerham yeast nitrogen base</td>
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I. INTRODUCTION

Dermatophytes are a group of taxonomically related fungi that infect man and other animals by the invasion of keratinized tissues, such as skin, nails, feathers and hairs. The infections produced are more or less superficial, not tending to involve the deeper tissue or to spread to the internal organs. Due to the variability shown by these organisms in artificial culture and the ability for different species to cause identical clinical infections on animals, the classification of the dermatophytes has caused a great deal of confusion and is still far from satisfactory. At present, dermatophytes are subdivided into three genera: Microsporum, Trichophyton and Epidermophyton, each of which can be distinguished morphologically by its macroscopic and microscopic features, pigment production and other physiological characters (Emmons et al., 1970).

Tate (1929) began one of the earliest study on the dermatophytes in this century. Extensive reviews dealing with various aspects of dermatophytes such as morphology (Langeron and Milochevitch, 1930), metabolism (Page, 1948), respiration studies (Nickerson and Chadwick, 1946), growth factors and nutritional requirements (Stockdale, 1953; Robbins and Ma, 1945), geographic distribution and prevalence (Ajello, 1960; Hagen, 1969) and various environmental factors affecting growth (Paldrok, 1955), have been published from time to time.

Electron microscopy of the dermatophytes has yielded limited information on their ultrastructures due primarily to technical difficulties (Blank et al., 1960; Urabe and Izu, 1969; and Akin and Michaels, 1972).
Trichophyton mentagrophytes, a species of the dermatophytes, causes several types of "tinea" in different parts of the body. The most common clinical feature of these, both in animals and man, is the penetration of hair and epidermis by the fungus, followed by the falling off of the infected tissues from the body. Although dermatomycoses were formerly very common diseases, their prevalence has been greatly decreased in recent years as a result of the introduction of the antibiotic, griseofulvin. Despite this improvement, dermatomycoses still remain as important epidemiological problems in communal life where high standards of personal hygiene are difficult to maintain. For instance, "athlete's foot" or tinea pedis, a most common disease caused by T. mentagrophytes, is still prevalent among coal miners and soldiers in many countries of the world. To date no effective prophylactic method is available.

Although it is known that vegetative hyphae are responsible for the erosion and penetration of the infected tissues (Davidson and Gregory, 1934), the role of spores in the pathogenicity and epidemiology of dermatomycoses is less well understood.

Two types of conidia are produced by T. mentagrophytes; these are the single-celled microconidia and the multi-celled macroconidia. Since microconidia are the most predominant form of T. mentagrophytes spores and are abundantly produced in most of the strains, the elucidation of conditions leading to the transformation of dormant conidia to infectious hyphae is undoubtedly of vital importance for understanding of roles of dermatophyte spores in the epidemiology of dermatomycoses.
The germination of bacterial spores has attracted the attention of numerous workers and extensive studies have been performed. The data accumulated by these workers are available in several reviews (Sussman and Halvorson, 1966; Halvorson et al., 1966; Gould, 1969). The term, germination, as applied to bacterial spores by Sussman and Halvorson (1966) refers to "a process which leads to the first irreversible change which is recognizably different from the dormant organism, as judged by physiological or morphological criteria". These criteria include loss of refractivity (darkening of the spore when examined under a phase-contrast microscope, Pulvertaft and Haynes, 1951; Powell, 1957; Campbell, 1957), increased stainability with basic dyes (Powell, 1950; Levinson and Sevag, 1953; Harrell and Halvorson, 1955) loss of resistance to heat and chemicals (Williams, 1952; Powell, 1957), reduction in optical density of the spore suspension (Powell, 1950), the release of dipicolinic acid, calcium and peptides containing muramic acid (Powell and Strange, 1953; Strange, 1959), enhanced respiratory activity (Mandels et al., 1956), and swelling and hydration of the spore (Lamanna, 1940). Most of these changes listed have been employed as parameters to follow the kinetics of bacterial spore germination. However, the loss of heat resistance, and the reduction of optical density is most frequently used in bacterial spore studies (Sussman, 1966).

In contrast to bacterial spores, germination of fungal spores is less clearly defined. Yanagita (1957) assessed the germination of Aspergillus niger conidia by following the changes in the conidial diameter, weight or
volume. Mandels and Darby (1953) and Terui and Mochizuki (1955) followed the decrease in light transmittance during conidial germination of *Microthecium verrucaria* and *Aspergillus oryzae*, respectively. Metabolic changes such as increase in respiratory activity (Mandels et al., 1956; Sussman, 1965), synthesis of total nitrogen, protein nitrogen and nucleic acid (Yanagita, 1957), and loss of heat resistance (Lingappa and Sussman, 1959) have also been employed in the assessment for various fungal spore germination. Germ tube protrusion has long been the most preferred and popular one employed by most of the workers because it apparently represents the earliest visible morphological change which could be detected by simple light microscopy. Since it is impossible to determine the exact moment at which germ tubes begin to protrude from the spore wall, difficulties again arise as to which stage of germ tube development should a spore be defined as germinated. For instance, it is recommended by the American Phytopathological Society (1943) that the spore is arbitrarily defined as germinated if the length of the tube exceeds half the minor diameter of the spore. However, several investigators, such as French (1961), Manners and Hossain (1963), prefer to define a spore as germinated when the germ tube is as long as it is broad. Moreover, most of the workers even failed to state what criteria they adopted for their assessment of germination (Calpouzos and Chang, 1972; Lösel, 1967; Gunasekaran et al., 1972). Such imprecise definition could result in incorrect conclusions making it virtually impossible to compare the results of different investigators studying the same organism. It appears imperative that a more rigorous definition be established for fungal spore germination.
Fundamentally, germination of fungal spores is the process involving the transformation of a resting spore into a metabolically active cell. Although this is a continuous process, it can be divided into a few distinct steps. Turian (1966), for example subdivided the process into three stages, i.e., the pregermination period, swelling phase, and the emergence of the germ tube, and Manners (1966) similarly divided the process into three phases, the internal changes within the spores, the protrusion of the germ tube and elongation of the tube. Although considerable variations in the morphological changes exist among different spores, the emergence of the germ tube is the most obvious event of fungal spore germination and is most frequently used as the criterion of germination. Swelling of spores as a result of intake of water was considered as the first visible sign of germination (Mandels and Darby, 1953; Yanagita, 1957). Alteration of shape and gain in stainability are also observed prior to germ tube emergence (Hawker, 1966).

It was not until the introduction of the electron microscope with its increased resolution that the ultrastructural changes during germination could be studied. Several representative works in this area include those of the sporangiospores of Rhizopus (Hawker and Abbott, 1963), wheat stem rust uredospores (Williams and Ledingham, 1964), the ascospores of Neurospora tetrasperma (Lowry and Sussman, 1968), the basidiospores of Psilocybe sp. (Stock and Hess, 1970), and the conidiospores of Botrytis cinerea (Buckley et al., 1966), Mucor rouxii (Bartnicki-Garcia et al., 1968), Aspergillus nidulans (Border and Trinci, 1970), Penicillium griseofulvum Dierckx (Fletcher, 1970), and Botrytis fabae Sardina (Richmond and Pring, 1971).
Changes in shape and numbers of mitochondria were reported to occur during the early stage of germination (Hawker and Abbott, 1963; Lowry and Sussman, 1968; and Fletcher and Morton, 1970). The endoplasmic reticulum, usually sparse or absent in the ungerminated spores, increased substantially during the early stage of germination (Williams and Ledingham, 1964; Buckley et al., 1966; and Fletcher, 1970). Protrusion of the germ tube is the most readily recognizable event among all the morphological changes that occur during germination. The origin of the germ tube wall is thought to be different in various species of spores (Sussman, 1966; Richmond and Pring, 1971; Hawker, 1966; and others). However, due to the diversity of the spore surface structure in different species of fungi, and also due to the limited number of reports describing the actual germination process, at this time no generalization can be made in this regard.

In addition to the morphological changes revealed by the electron microscope, there has accumulated a considerable knowledge of the changes in physiological properties of spore during germination. Major contributions have been made by Yanagita (1957), Cochrane and co-workers (1963), Allen (1965), and others on various fungus spores. A marked increase in the respiratory activity was first reported in germinating ascospores of Neurospora tetrasperma (Goddard, 1935). Similar results were also reported with conidia of *Aspergillus niger* (Yanagita, 1957), *Myrothecium verrucaria* (Mandels, et al., 1956) and others. However, a drop in respiratory rate was sometimes observed as in uredospores of rust fungi (Shu et al., 1954) and zoospores of *Blastocladiella* (Cantino and Lovett, 1960). Cantino and Lovett (1960), Sussman (1966), and Cochrane and Cochrane (1966) reported
that most enzymes concerned with respiration showed an increased specific activity during germination. Utilization of spore reserves, alterations in nucleic acids and nucleoproteins, and synthesis of macromolecules were also observed to occur during germination of fungal spores (Allen, 1965; and Brambl and Van Etten, 1970).

The effect of temperature on fungal spore germination was reviewed by Goddard (1935), Gottlieb (1950) and Sussman (1966). Although it is generally recognized that fungi prefer aerobic conditions for spore germination, a limited rate of germination under anaerobic condition has been reported with spores of certain species of fungi (Wood-Baker, 1955; and Cochrane et al., 1963).

Carbon dioxide, an essential factor for the germination of certain bacterial spores, also promotes the germination of the conidia of Aspergillus niger (Yanagita, 1957). Vakil et al. (1961) reported that the effect of carbon dioxide is more pronounced at low spore densities and at low pH. Fixation of carbon dioxide has also been reported for spores of other fungi (Kosuge and Dutra, 1963, Phytopathology Abstr. 53:880), but in most instances it has not been shown to promote germination.

Other factors such as temperature, hydrogen ion concentration, relative humidity (Sussman, 1966), light, and various chemicals are also reported to influence the germination of fungal spores (Sussman, 1965). Treatment of spores with a sublethal dose of heat has been frequently used to stimulate bacterial spore germination. However, the need of such heat treatment for activation of fungal spores is reported only in the ascospores of certain Ascomycetes (Sussman, 1966).
Dormancy of fungus spores can be broken by treatment with a variety of chemicals. Alcohols, ketones, chloroform, ethyl ether and other solvents have been found to activate the germination of certain fungal spores (Sussman, 1966). For instance, pretreatment with very low concentration of furfural or some other heterocyclic compounds stimulates the germination of certain fungal spores (Lingappa and Sussman, 1959). In addition to these, naturally occurring compounds such as esters, indoleacetic acids, lactones, organic acids, amino acids and vitamins are also found to stimulate germination of fungal spores (Sussman, 1966).

A simple compound such as L-alanine or adenosine is essential for triggering the germination of certain bacterial spores (Powell and Hunter, 1955; Powell, 1950; and others). Meso-inositol, niacinamide and thiamine have been reported to stimulate fungal spore germination (Sussman, 1966). Recent studies demonstrated that a single amino acid such as L-alanine induced the germination of Aspergillus niger conidia (Yanagita, 1957) and L-proline induced germination of the sporangiospores of Rhizopus arrhizus and Rhizopus stolonifer (Weber, 1962; Weber and Ogawa, 1965). However, the role of some of these substances has to be interpreted with caution since it is not clear whether they are required as activators or for mycelial development since germ tube emergence was employed as the criterion of germination in all of the studies reported above.

In spite of advances made in the field of bacterial and fungal spore germination in recent years, information on the requirements for germination of dermatophyte spores is very limited. The only dermatophyte spore system
reported to date is that of the macroconidial germination in *Microsporum gypseum* (Barash *et al.*, 1967; Leighton and Stock, 1970).

The investigation reported in this thesis was undertaken in order
a) to elucidate morphological and physiological changes associated with the germination of *T. mentagrophytes* microconidia, b) to elucidate the specific nutritional requirements and physiological conditions for the microconidial germination, and c) to investigate the physiological and chemical factors affecting the *T. mentagrophytes* microconidial germination.
II. MATERIALS AND METHODS

Organism. *Trichophyton mentagrophytes* strain SF 306 A/68, obtained from Dr. J. Rippon of the University of Chicago, was used in this study.

Maintenance of the organism. Stock cultures were maintained at room temperature on Difco Sabouraud glucose agar (SGA) with periodical transfers of the granular type colonies to avoid the development of pleomorphic forms of the fungus.

Cultivation of the fungus. Modified Difco SGA (2% glucose, 1% peptone, 1.5% bacto-agar, pH 7.0) was used as the sporulation medium. Large quantities of microconidia were produced on this medium after 4 to 7 days of incubation at 30°C.

Preparation of the microconidial suspension. Microconidia were dislodged from the agar surface (7 to 10 days old culture) by scraping gently with a sterile policeman made of glass rod covered with a piece of tygon tubing. The conidia collected from 1 to 2 plates were then transferred to a sterile test tube (25 X 100 mm) containing 1 ml of sterile water and gradually were made into a homogenous suspension by gently rubbing the spores with the policeman against the test tube wall. Filtration of the conidial suspension through ten layers of sterile cheesecloth proved useful in eliminating hyphae and clumped masses of conidia. Microconidia in the filtrate were washed in cold distilled water five to eight times until a microscopically clean conidial suspension was obtained. The washed microconidia were dispensed in small vials and kept frozen at
-10 C until use. Microconidial preparations showing less than 10% of phase dark conidia after incubation in sodium phosphate buffer (0.1 M, pH 6.0) at 37 C overnight were considered pure and used in our experiments.

Determination of viable cell counts. The concentration of the microconidial suspension was routinely determined turbidimetrically in a Klett-Summerson photoelectric colorimeter (model 800-3, Klett Mfg. Co., New York) equipped with a No. 54 green filter (540 nm) using a predetermined standard chart (Fig. 1). Viable conidial counts were determined by the standard plating technique. A conidial suspension of known optical density was serially diluted in sterile distilled water and 0.1 ml samples were plated out in duplicate, by means of a bent glass rod, on SGA plates. Colonies which developed after 3 to 4 days of incubation at 30 C were counted.

Phase contrast microscopy. The changes in refractility of microconidia during germination was observed in a dark phase contrast microscope by using an oil immersion objective (DM 100 X; numerical aperture, 1.25; Nikon) and photomicrographs of the conidia were taken with a Nikon M-35S camera attached to the microscope using Plus-X film (Eastman Kodak Co., Rochester, New York). Unless otherwise stated, all phase contrast micrography was made using wet mount preparations.

Definition and assessment of germination. In the present study, microconidia were considered germinated when loss of refractility
Fig. 1. Standard chart illustrating Klett units versus viable counts of *T. mentagrophytes* microconidia. The concentration of the microconidial suspension was determined turbidimetrically in a Klett-Summerson photoelectric colorimeter equipped with a No. 54 filter (540 nm). Viable counts were determined by the standard plating technique as described in the Materials and Methods section.
became evident under a phase contrast microscope (Fig. 2a to c). The percent of germination in a given sample was calculated by counting the numbers of germinated microconidia out of 200 randomly selected conidia under the microscope.

**Preparation of thin section for electron microscopy.** Samples were first fixed with glutaraldehyde (4% in veronal acetate buffer, pH 6.0) for 6 hr at 20 C. After washing in the buffer twice, the cells were fixed with 2% osmium tetroxide (Kellenberger et al., 1959) overnight at 20 C and then treated with uranyl acetate (0.5%) for 1 hr. After dehydration through a graded acetone series the cells were embedded in a mixture of Epon 812 and 815 and polymerized at 60 C for 22 hr. Sections were cut in an LKB 4800A ultratome (LKB-Produkter AB, Stockholm, Sweden) using a glass knife and mounted on formvar coated copper grids (300 mesh, Ernest F. Fullam, Inc., Schenectady, New York). After staining with lead citrate (Reynolds, 1963), sections were viewed with a Hitachi HU-11A electron microscope operating at 75 kV.

**Resistance to stain with basic dye.** Dormant and germinated microconidia were smeared on clean glass slides and heat-fixed. The preparations were then flooded with 0.1% Loeffler's methylene blue and kept at room temperature for 10 min. Those microconidia which failed to stain with methylene blue within 10 min were considered resistant to the stain.

**Lipid stain.** For the observation of lipid or fatty materials within the microconidia the Burdon's method (Burdon, 1946) was employed. Staining
solution was prepared by dissolving 0.3 g of Sudan black B (E.H. Sargent & Co., Detroit, Michigan) in 100 ml of 70 % ethyl alcohol. Dormant or germinated T. mentagrophytes microconidia were smeared on clean glass slides and dried at room temperature. After brief fixation with heat, the slides were flooded with the staining solution and allowed to stand at room temperature for 10 to 15 min. Excess stain was drained and slides were blot dried. After being covered with xylene and blotted dry, the slides were mounted with a drop of water, and the edges of a cover glass were sealed with vaspar (1:1 mixture of vaseline and paraffin) prior to examination under a light microscope.

Microculture of T. mentagrophytes. A few drops of sterile molten SGA were placed on a clean sterile glass slide (3" X 1") and immediately covered by a sterile cover glass (22 X 22 mm, No. 1 thickness). After solidification of the agar, the cover glass was removed and the slide was point-inoculated with the microconidia. Two capillary tubes (5 to 8 mm long, 0.1 to 0.5 mm diameter) were placed on each side of the agar and then covered with a sterile cover glass. The slide was then sealed with vaspar, placed in a petri dish containing moistened filter paper and incubated at 37 C. Time lapse photomicrography of the germination process was carried out under a phase contrast microscope as described above.

Manometric measurements. Oxidation of glucose by dormant and germinated microconidia was studied by standard manometric techniques (Umbreit, 1964). Germinated microconidia were prepared by inoculating 5 ml of
dormant microconidia \((3.0 \times 10^7 \text{ cells/ml})\) into 5 ml of 2 \% peptone solution in a 250 ml Erlenmyer flask and incubated at 37 C on a rotary shaker (model G-10, 220 rev/min, New Brunswick Scientific Co., N. J.). Cultures were then harvested, washed twice with sodium phosphate buffer (0.1M, pH 7.0) and resuspended in 5 ml of the same buffer. A 1 ml sample of dormant or germinated microconidia \((3.0 \times 10^7 \text{ cells/ml})\) was then placed in the main compartment of the Warburg vessel. The center well contained 0.2 ml of 40 \% KOH solution and 1 ml of glucose solution (100 mM) was added into the side arm of the flask. For the endogenous control, 1 ml of distilled water was added in place of glucose. The gaseous phase was air. After 10 min of equilibration at 37 C, the glucose solution was tipped into the reaction chamber. Readings were taken every 15 to 30 min interval over a 2 hr period. \(Q_02\) values were calculated from data obtained during the first 120 min and were expressed as \(\mu l\) of \(O_2\) consumption per \(1.5 \times 10^7\) cells.

**Basic germination system.** Unless otherwise stated, small tubes (22 mm X 75 mm) containing 1 ml of *T. mentagrophytes* microconidial suspension \((5 \times 10^6 \text{ cells/ml})\) were incubated at 37 C in the presence of an appropriate germinant on a rotary shaker (model G-10, 220 rev/min, New Brunswick Scientific Co., N. J.) under specified conditions. The system was buffered to pH 6.0 with a sodium phosphate buffer (0.1 M). Samples were removed at appropriate intervals for the microscopical determination of percentage of germination. In most experiments L-leucine, at a concentration of 25 \(\text{mM}\), was used as the germinant.
Dry weight determination. Samples containing 5 ml of a microconidial suspension (in water) of known concentration were transferred into aluminum weighing dishes and dried at 90°C to a constant weight. All weighing dishes were dried to a constant weight prior to usage.

Cell size determination. A computerized cell counter, Electrozone Celloscope (Particle Data, Inc., Elmhurst, Ill.) was employed to determine the average diameter of both dormant and germinated microconidia. For each measurement a minimum of 25,000 cells were counted.

Separation of dormant and germinated microconidia by linear Renografin density gradient centrifugation. In order to study the change in conidial density before and after germination, a modified method of Tamir and Gilvarg (1966) was used. Linear Renografin (Reno M-60, meglumine diatrizoate, E. R. Squibb & Sons, Inc., New York) gradients were formed by means of a two cylinder plexiglass gradient-maker. The gradient formed from the gradually diluted Renografin was collected in a polyallomer tube (IEC) at a rate of one drop/sec. The Renografin gradient was used immediately upon formation to minimize diffusion effects. A 0.5 ml sample of conidial suspension was layered on top of the gradient and the tubes containing the gradients were then centrifuged in an IEC ultracentrifuge (model MB 35, International Equipment Co., Needham Hts., Mass.) in a swinging bucket rotor (SB-269) at 4°C for 30 min at 17,000 rev/min. The individual bands formed in the gradient were withdrawn carefully by a syringe and diluted 1 to 10 with water. The microconidia were washed with
sterile distilled water and examined for their viability by incubating
them overnight at 37 C in 1 % peptone.

**Heat activation.** To test the effect of sublethal heat treatment on
L-leucine induced germination, microconidia were pretreated at 40 C and
45 C for varying lengths of time before being mixed with L-leucine.

**Chemicals.** All of the amino acids and dipeptides were purchased as
chromatographically pure from Sigma Chemical Co., St. Louis, Mo. Unless
specifically mentioned otherwise, all amino acids and dipeptides refer to
the L isomers. The purines, pyrimidines, griseofulvin, and phenylmethyl-
sulfonyl fluoride (PMSF) were also purchased from Sigma Chemical Co.,
whereas carbohydrates and other biochemicals were from Nutritional
Biochemical Co., Cleveland, Ohio. The polyene antibiotics were purchased
from Upjohn Co., Kalamazoo, Mich. All other chemicals were of reagent
grade.
III. RESULTS

A. Search for germination inducer(s) of *T. mentagrophytes* microconidia.

The dormant microconidia of *T. mentagrophytes* lost their refractility within 2 hr when incubated in a complex medium (SGA or SGB) at 37 C (Fig. 2a to c). As the initial step to identify the materials essential for germination, the microconidia were germinated in peptone (1 %) in combination with varying glucose concentrations (0.1 % to 4 %). Results (data not shown) indicated that glucose was not necessary for germination while peptone alone at a concentration as low as 0.1 % could induce germination of *T. mentagrophytes* microconidia. Dialysis of peptone through a cellophane membrane resulted in the separation of the active principle(s) in the dialysate whose activity was not affected by autoclaving. It was simultaneously found that germination of *T. mentagrophytes* microconidia occurred in a synthetic medium containing 19 amino acids supplemented with yeast nitrogen base (YNB, Wickerham and Burton, 1948) (Table 1). Through the process of elimination, it was found that the active principles for germination in this synthetic medium was the amino acid solution. As shown in Table 2, each of the following amino acids, L-alanine, L-leucine, L-isoleucine, L-methionine, L-tryptophan, L-valine, and glycine, was able to induce germination of *T. mentagrophytes* microconidia. Among these 7 amino acids, L-leucine proved to be the most effective germination inducer (Table 2). No germination was observed in water, sodium phosphate buffer or any other buffer solutions.

Germination inducer other than L-leucine. A number of nitrogenous compounds, organic acids, and carbohydrates were tested for their ability to induce
Table 1.

The composition of the synthetic medium used for the germination of T. mentagrophytes microconidia.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>mg/l</th>
<th>Amino acid</th>
<th>mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Arginine</td>
<td>105.0</td>
<td>L-Threonine</td>
<td>48.0</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>24.0</td>
<td>L-Tryptophan</td>
<td>10.0</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>31.0</td>
<td>L-Alanine</td>
<td>890.0</td>
</tr>
<tr>
<td>L-Valine</td>
<td>46.0</td>
<td>L-Asparagine</td>
<td>1500.0</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>292.0</td>
<td>L-Aspartic acid</td>
<td>1330.0</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>52.5</td>
<td>L-Glutamic acid</td>
<td>1470.0</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>52.4</td>
<td>L-Proline</td>
<td>1150.0</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>58.0</td>
<td>L-Serine</td>
<td>1050.0</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>15.0</td>
<td>Glycine</td>
<td>750.0</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>32.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Wickerham's yeast nitrogen base.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>µg/l</th>
<th>Ingredients</th>
<th>µg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biotin</td>
<td>2</td>
<td>Copper sulfate</td>
<td>40</td>
</tr>
<tr>
<td>Calcium pantothenate</td>
<td>400</td>
<td>Potassium iodide</td>
<td>100</td>
</tr>
<tr>
<td>Folic acid</td>
<td>2</td>
<td>Ferric chloride</td>
<td>200</td>
</tr>
<tr>
<td>Inositol</td>
<td>2000</td>
<td>Manganese sulfate</td>
<td>400</td>
</tr>
<tr>
<td>Niacin</td>
<td>400</td>
<td>Sodium molybdate</td>
<td>200</td>
</tr>
<tr>
<td>p-Amino benzoic acid</td>
<td>200</td>
<td>Zinc sulfate</td>
<td>400</td>
</tr>
<tr>
<td>Pyridoxine hydrochloride</td>
<td>400</td>
<td>Potassium phosphate monobasic</td>
<td>1</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiamine hydrochloride</td>
<td>400</td>
<td>Magnesium sulfate</td>
<td>0.5</td>
</tr>
<tr>
<td>Trace elements</td>
<td></td>
<td>Sodium chloride</td>
<td>0.1</td>
</tr>
<tr>
<td>Boric acid</td>
<td>500</td>
<td>Calcium chloride</td>
<td>0.1</td>
</tr>
</tbody>
</table>
TABLE 1 (CONT'D.)

a Stock solution of amino acids and bacto-yeast nitrogen base were separately prepared and sterilized by membrane filtration (0.45 µm pore size, Millipore Filter Co., Bedford, Mass.). Stock solutions were then diluted in sterile distilled water to give desired concentrations.
Table 2.

Germination of *T. mentagrophytes* microconidia
induced by L-amino acids\(^a\).

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Germination (^b)</th>
<th>Amino Acid</th>
<th>Germination (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>30-60</td>
<td>Leucine</td>
<td>60-95</td>
</tr>
<tr>
<td>Arginine HCl</td>
<td>&lt; 10</td>
<td>Lysine hydrochloride</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>Asparagine</td>
<td>&lt; 10</td>
<td>Methionine</td>
<td>30-60</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>&lt; 10</td>
<td>Phenylalanine</td>
<td>10-30</td>
</tr>
<tr>
<td>Cysteine</td>
<td>&lt; 10</td>
<td>Proline</td>
<td>10-30</td>
</tr>
<tr>
<td>Cystine(^c)</td>
<td>&lt; 10</td>
<td>Serine</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>&lt; 10</td>
<td>Threonine</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>Glutamine</td>
<td>&lt; 10</td>
<td>Tryptophan</td>
<td>30-60</td>
</tr>
<tr>
<td>Histidine hydrochloride</td>
<td>&lt; 10</td>
<td>Tyrosine(^c)</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>Hydroxy-L-proline</td>
<td>&lt; 10</td>
<td>Valine</td>
<td>30-60</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>30-60</td>
<td>Glycine</td>
<td>10-30</td>
</tr>
</tbody>
</table>

\(^a\) Individual amino acid was added at final concentration of 25 mM in small tubes (22 x 78 mm) containing 5.0 x 10^6 microconidia/ml, buffered at pH 6.0 (0.1 M, sodium phosphate buffer). All tubes were incubated at 37°C on a rotary shaker.

\(^b\) Germination percentages were estimated microscopically as described in the materials and methods section.

\(^c\) Saturated solutions were used due to the limited solubility of these compounds.
germination of *T. mentagrophytes* microconidia. No compounds other than certain amino acids (Table 2) and dipeptides (Table 8) were capable of causing germination of the microconidia 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 the L-leucine-induced germination of the microconidia.

**Carbohydrates and organic acids.** Carbohydrates and organic acids (0.5 % in sodium phosphate buffer, 0.1 M, pH 6.0) were as follows: D-glucose, D-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-mannosamine, and D-galactosamine hydrochloride.

**Purines, pyrimidines, and nucleosides.** Purines, pyrimidines, and nucleosides (20 mM in 0.1 M sodium phosphate buffer, pH 6.0) were as follows: adenine, cytosine, guanine, uracil, xanthine, hypoxanthine, adenosine, cytidine sulphate, 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.
B. Changes associated with germination of *T. mentagrophytes* microconidia.

1. Cytological changes

**Light microscopic changes.** The most simple means to differentiate the dormant microconidia from the germinated ones was the use of a dilute solution of a basic dye. The dormant conidia were resistant to staining with 0.1% methylene blue while the germinated phase-dark conidia were not. It was also shown that large lipid bodies occupying a considerable portion of the dormant conidia (Fig. 2f) underwent fragmentation during germination and were eventually translocated to the periphery of the conidia (Fig. 2g). These granules completely disappeared when the germinated conidia reached the stage of forming short germ tubes (Fig. 2d).

Phase contrast microscopy of *T. mentagrophytes* microconidia undergoing germination at 37 °C in a complex medium revealed that the dormant conidia lost their refractility and became phase dark within 2 hr (Fig. 2c). Emergence of germ tube took place in 3 hr (Fig. 2d). These tubes then elongated and eventually became the vegetative hyphae (Fig. 2e). When microconidia were germinated by L-leucine only, they became phase dark within 6 to 8 hr. Short germ tubes usually emerged from the conidial surface when such conidia were further incubated for a prolonged period (22 hr). These tubes normally never exceeded one half of the average diameter of the microconidia (Fig. 2d).

In order for subsequent vegetative outgrowth to occur (Fig. 2e), the supplementation of additional nutrients was essential. It should be emphasized that when germinated phase-dark conidia (Fig. 2c) were transferred into sodium phosphate buffer (pH 6.0, 0.1 M) or water after several
Fig. 2 a-e. Phase contrast micrographs showing the transformation of dormant *T. mentagrophytes* microconidia into vegetative hyphae. A dormant microconidium (2a) gradually loses its refractility (2b) and becomes fully phase dark (2c) within a short period of time (2 hr in 0.5 % peptone broth and 6 to 8 hr in 25 mM L-leucine). Germ tubes usually emerge (2d) at the end of 3 to 22 hr in SGB or L-leucine, respectively. Subsequent elongation and outgrowth of these tubes can further take place in the presence of exogenously added nutrients (2e).

Fig. 2 f-g. Light micrographs of *T. mentagrophytes* microconidia stained with Sudan black B. Note the large lipid bodies that occupy a considerable portion of the dormant conidia (2f), and the disappearance of these large lipid bodies and their transformation into smaller granules located near the peripheral region of the germinated conidia (2g).
In germination, the conidial wall ruptured and the conidial wall mesosomes showed a complete replacement. During germination, the conidial wall mesosomes and emerging germ tubes were fully encapsulated within the germination tube. In the complete absence of exogenous nutrients, the conidiophore filament was fully formed. The cell structure shown in Fig. 2a, 2b, 2c, 2d, 2e, 2f, and 2g appeared electron-dense (215 to 220 nm thick) and surrounded by a thin cytoplasmic layer (25-27 nm), and a nucleus  

The 10 μm scale bar indicates the relative size of the structures observed.
washings, they were still able to form short germ tubes. Apparently, germinated microconidia were fully capable of developing short germ tubes in the complete absence of exogenous nutrients.

_Ultrastructural changes as revealed by electron microscopy._ The transition from a dormant to a germinated microconidium involves changes in wall structure and the reorganization of various cytoplasmic organelles. As shown in Fig. 3, the dormant microconidial wall is found to consist of at least two layers. The outer wall (OW) is relatively thin (25 to 27 nm) and appears electron-dense, whereas the inner wall (IW) is relatively thick (225 to 250 nm) and composed of materials of low electron density. The cytoplasm of a dormant conidium contains large lipid granules (L) surrounded by a single electron dense layer, relatively few mitochondria (M), and a nucleus (N) with a nuclear membrane (NM).

In germinated microconidia (Fig. 4), mitochondria (M) assumed a more irregular or lobular appearance. With the technique used, no alterations in the structure of the nucleus (N) were noted and the nuclear membrane (NM) persisted throughout the whole germination process. The most striking change observed in the conidia as a result of germination was the almost complete replacement of the large lipid bodies with vacuoles (V) (Fig. 5).

During germ tube protrusion (Fig. 5), the outer conidial wall (OW) was ruptured and the new germ tube wall (VW) emerged from the inner layer of the conidial wall (IW). Membranous organelles similar to bacterial mesosomes are often observed within the cytoplasm of germinated microconidia with emerging germ tubes.
Fig. 3. Electron micrograph of an ultrathin section of a dormant T. mentagrophytes microconidium. The outer wall (OW) is relatively thin and appears electron-dense. A thick inner wall (IW) is electron transparent. Large lipid bodies (L), and a mitochondrion (M) can also be seen near the nucleus (N).

Fig. 4. Electron micrograph of an ultrathin section of a germinated T. mentagrophytes microconidium. Mitochondria (M) appear irregular and the cristae are more distinctly visible. Note that the large lipid bodies (L) seen in the dormant microconidium (Fig. 3) underwent fragmentation into smaller granules (L).
Fig. 5. Electron micrograph of an ultrathin section through a germinating microconidium showing the emergence of a germ tube. Rupturing of the outer conidial wall (OW) is apparent. The newly formed germ tube wall (VW) is evidently continuous with the innermost wall (IW) of the dormant conidium. Elongated or lobate mitochondria are visible (M). Nucleus (N) and fragmented vacuoles (V) are also shown.
2. Changes in physical properties.

Cell size. The germinated microconidia, when examined under a light microscope, appeared larger in size due to the swelling. The analysis of cell size by an Electrozone Celloscope revealed that there was an increase in conidial diameter (approximately 10%) after germination (Table 3).

Dry weight. The reduction in dry weight, approximately 9%, occurred after the germination of microconidia (Table 3).

Density. The complete separation of dormant and germinated microconidia was achieved through the use of Renografin density gradient centrifugation. As shown in Fig. 6, when a mixture of dormant and germinated microconidia was layered on a gradient, two distinctive bands were observed upon centrifugation (Fig. 6). Microscopic observation showed that the lower band (arrow 2) contained the dormant, refractile conidia, whereas the upper band (arrow 1) consisted of the germinated, phase-dark conidia. This indicates that the reduction in the specific gravity of the microconidia occurs after germination.

Our preliminary tests showed that exposure to Renografin had no detrimental effects on either dormant or germinated microconidia of T. mentagrophytes.
TABLE 3.

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

<table>
<thead>
<tr>
<th>Conditions of Microconidia</th>
<th>Dry wt (mg) 1.5 x 10^8 Microconidia</th>
<th>Cell diameter</th>
<th>Glucose oxidation (counts/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total no. counted</td>
<td>Mean (µm)</td>
<td>Glucose-1-^{14}C</td>
</tr>
<tr>
<td>----------------------------</td>
<td>--------------------</td>
<td>-----------</td>
<td>----------------</td>
</tr>
<tr>
<td>Dormant</td>
<td>4.06</td>
<td>27,691</td>
<td>3.52 ± 0.26</td>
</tr>
<tr>
<td>Germinated^b</td>
<td>3.71</td>
<td>30,735</td>
<td>3.88 ± 0.18</td>
</tr>
</tbody>
</table>

^a Measured as ^14^CO₂ evolved from glucose-1-^{14}C or -6-^{14}C during 4 hr.
Each reaction flask contained 2.5 ml of conidial suspension (3 x 10^7 cells/ml), 1 ml of sodium phosphate buffer (0.1 M, pH 6.0), and 0.5 ml of ^14^C-labeled glucose solution (25 µmoles) 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 (1965). The data presented were obtained from the experiments carried out by R. Belkengren.

^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-conidial suspensions were used in the experiments.
Fig. 6. Separation of dormant and germinated microconidia of *T. mentagrophytes* after linear Renografin density gradient centrifugation. Tube A contains a mixture of dormant and germinated microconidia, tube B contains the dormant conidia only, and tube C contains the germinated conidia only. The gradient range of Renografin is 24 % at the top and 48 % at the bottom.
3. **Physiological changes.**

**Respiration.** Data obtained from Warburg respirometer studies (Fig. 7) show that the dormant microconidia utilized very little oxygen in the presence of D-glucose (DG). The germinated conidia not only exhibited a significantly higher endogenous respiration (GE), but also a marked increase in \( O_2 \) consumption after a lag of 35 min in the presence of exogenous D-glucose (GG).

**Heat resistance.** Fig. 8 represents the thermal inactivation curve of both dormant and germinated conidia exposed to 55 °C. It is apparent that microconidia lost their resistance to heat upon germination. The simultaneous loss of both viability and germinability were observed when dormant microconidia were exposed to lethal doses of heat.
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 (2.0 x 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. 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. The conidia were treated at 55 C for specified periods and the viability of the heated conidia were determined as described in Materials and Methods. The loss of germinability on leucine was determined microscopically after incubation of conidia for 24 hr at 37 C in the presence of L-leucine (25 mM in phosphate buffer, 0.1 M, pH 6.0).
C. Factors affecting leucine-induced *T. mentagrophytes* microconidial germination.

Microconidia of *T. mentagrophytes* were found to germinate in a simple chemically defined system containing L-leucine. The effects of several physical or chemical factors influencing the leucine-induced germination system were studied in detail.

**Effect of leucine concentration.** When a moderate concentration of microconidia (5 \( \times 10^6 \) cells/ml) was used, the leucine concentration affected the rate but not the extent, of germination within the range of leucine concentrations tested (Table 4). Complete germination was achieved within 18 hr at a leucine concentration as low as 0.1 mM when 5 \( \times 10^6 \) conidia/ml were used. However, 25 mM was chosen as the optimal leucine concentration for all the subsequent germination studies.

**Effect of pH and buffers.** Five different types of buffers were used in preliminary studies. Since germination of the microconidia was poor at pH 4.0 and 7.5, citrate phosphate buffer (effective range, pH 4.6 to 6.6) and sodium phosphate buffer (effective range, pH 6.0 to 7.5) were selected to determine the optimal pH for germination. As shown in Fig. 9, the optimal pH appears to be between 6.0 to 6.5. Sodium phosphate buffer alone did not induce germination of the microconidia, nor did it have any effect on the leucine-induced germination (Table 5).

**Effect of inoculum size.** Because the extent of bacterial spore germination is influenced by the spore concentration in the germination medium,
Effect of L- and D-leucine concentration on the germination of heat-activated microconidia of *T. mentagrophytes*.

<table>
<thead>
<tr>
<th>Conc (mM)</th>
<th>Germination (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L-Leucine</td>
</tr>
<tr>
<td></td>
<td>4 hr</td>
</tr>
<tr>
<td>25</td>
<td>82</td>
</tr>
<tr>
<td>10</td>
<td>68</td>
</tr>
<tr>
<td>1</td>
<td>64</td>
</tr>
<tr>
<td>0.1</td>
<td>54</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percent of germinated spores after incubation at 37 °C and 4 hr, 8 hr 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 x 10<sup>6</sup> spores in 1 ml of phosphate buffer (0.1 M, pH 6.0) containing the specified concentration of the germinating agent.
TABLE 5.

Effect of sodium phosphate buffer concentrations on L-leucine induced germination of *T. mentagrophytes* microconidia.

<table>
<thead>
<tr>
<th>Sodium phosphate buffer (pH 6.0) concn (M)</th>
<th>Germination (%) (5 1/2 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 x 10^{-1}</td>
<td>&gt; 95 %</td>
</tr>
<tr>
<td>5.0 x 10^{-2}</td>
<td>&gt; 95 %</td>
</tr>
<tr>
<td>2.5 x 10^{-2}</td>
<td>&gt; 95 %</td>
</tr>
<tr>
<td>1.25 x 10^{-2}</td>
<td>&gt; 90 %</td>
</tr>
</tbody>
</table>

\*a\ Percent of germination after 5 1/2 hr of incubation in the basic germination system at 37 C. The concentration of microconidia in all tubes was 5 x 10^6/ml.
the effect of inoculum sizes on the leucine-induced germination of *T. mentagrophytes* microconidia was studied. Data presented in Table 6 indicates that there was an inverse relationship between the microconidia concentration and their extent of germination. As the inoculum size increased from $3.4 \times 10^6$ cells/ml to $1.5 \times 10^7$ cells/ml, a gradual drop in germination percentages (for instance, from 89% to 48% at the end of 9 hr incubation period) was observed. A significant inhibition occurred when the microconidial concentration exceeded a level of $10^7$ cells/ml. It is apparent that crowding reduces the extent of germination of the microconidia.

**Effect of sublethal heat.** Previous studies showed that microconidia lost their germinability when treated at 55°C for 5 min (Fig. 8). However, the treatment of the conidia with sublethal doses of heat (40°C and 45°C) for varying periods of time prior to the addition of L-leucine or peptone broth (0.5%) markedly increased the germination rate (Fig. 10). The heat-shocked microconidia had a similar microscopic appearance as compared with the untreated conidia.

**Effect of carbon dioxide and oxygen.** The effect of CO$_2$ on microconidial germination was studied in two different ways. When studied by the microculture technique, the presence of CO$_2$ appeared to have a slight stimulating effect on the initial phase of microconidial germination. In the complete absence of CO$_2$, the initiation of microconidial germination was slightly delayed (Fig. 11). However, when this effect was studied by the Thunberg tube technique, no significant difference was noted in the rate or extent of germination within the presence or absence of CO$_2$. 
TABLE 6.

Effect of inoculum size on leucine-induced germination of *T. mentagrophytes* microconidia

<table>
<thead>
<tr>
<th>No. of spores/ml (x 10^6)</th>
<th>Germination (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 hr</td>
</tr>
<tr>
<td>15.2</td>
<td>30</td>
</tr>
<tr>
<td>10.8</td>
<td>40</td>
</tr>
<tr>
<td>8.2</td>
<td>35</td>
</tr>
<tr>
<td>6.2</td>
<td>49</td>
</tr>
<tr>
<td>4.5</td>
<td>38</td>
</tr>
<tr>
<td>3.4</td>
<td>42</td>
</tr>
</tbody>
</table>

<sup>a</sup> 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.
Fig. 9. 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 x 10^6 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. 10. Germination curves of *T. mentagrophytes* microconidia (5 x 10^6 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.
Although the data are not shown here, rapid germination of the microconidia occurred in Thunberg tubes in the almost complete absence of oxygen (N₂ gas, 100 %) or in vacuum.

**Effect of incubation temperature.** The effect of incubation temperatures on leucine-induced germination is shown in Fig. 12. A sharp increase in germination occurred at a temperature above 30 °C with the optimal observed at 37 °C. At 45 °C, less than 45 % germination was obtained after 6 hr of incubation and no further increase was observed even after a prolonged period of incubation. These microconidia germinated at 45 °C did not lose the refractility completely. The optimum temperature for germination of *T. mentagrophytes* microconidia in 0.5 % peptone was also 37 °C.

**Effect of age of the microconidia on germination.** As shown in Table 7, microconidia harvested from different culture plates sporulated for varying lengths of time did not germinate similarly. The older the conidia were, the longer the lag phase they displayed. Conidia obtained from cultures ranging from 7 to 24 days of incubation, however, germinated more or less in the same fashion. The prolonged aging (36 days) seem to have a detrimental effect on the germinability of *T. mentagrophytes* microconidia. A large percentage of the excessively aged conidia (approximately 50 %) appeared to completely lose their germinability. Regardless of the age, the microconidia germination was significantly stimulated by sublethal heat treatment.
Fig. 11. Effect of carbon dioxide on the germination of *T. mentagrophytes* microconidia. All experiments were carried out in Thunberg tubes as described in Materials and Methods. The gaseous states were (○), carbon dioxide (95% CO₂ and 5% N₂); (△), atmosphere (0% CO₂, 80% N₂, and 20% O₂); (□) Vacuum.

Fig. 12. Effects of temperature on leucine-induced germination of microconidia and on vegetative growth of *T. mentagrophytes*. The germination system contained 5 x 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 7.

Effect of microconidial age on leucine-induced germination of *T. mentagrophytes*

<table>
<thead>
<tr>
<th>Microconidial age (day)</th>
<th>Germination&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>4 hr</td>
<td>8 hr</td>
<td>22 hr</td>
<td>Heat-activated&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4 hr</td>
</tr>
<tr>
<td>7</td>
<td>48 62 71</td>
<td></td>
<td></td>
<td></td>
<td>59 80 91</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>39 73 81</td>
<td></td>
<td></td>
<td></td>
<td>70 93 95</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>49 69 81</td>
<td></td>
<td></td>
<td></td>
<td>62 84 90</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>18 28 56</td>
<td></td>
<td></td>
<td></td>
<td>14 22 73</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Germination percentages were estimated microscopically as previously described at the end of 4, 8 and 22 hr incubation period.

<sup>b</sup> Microconidia were heat-activated at 45 C for 30 min before inoculating into the basic germination system.
Effect of dipeptides on the leucine-induced germination. Among the dipeptides listed in Table 8, leucyl-valine (25 mM) caused germination of the microconidia at a rate comparable to that obtained by leucine alone. Peptides containing L-leucine as the N-terminal amino acid such as leucyl-leucine and leucyl-tyrosine seemed to be more effective inducers than those with other N-terminal amino acids. No germination took place in the presence of valyl-tyrosine, valyl-valine and N-carbamyl-L-leucine.

Effect of fatty acids and of leucine analogs on the leucine-induced germination. No fatty acids were able to induce germination independently. With the exception of myristic acid (C\textsubscript{14}), most fatty acids at a concentration of 10 mM inhibited germination induced by L-leucine (Table 9). The same fatty acids at a concentration of 10 mM also suppressed germination of the microconidia in 0.5 % peptone broth.

None of the leucine analogs listed in Table 9 served as germinants of \textit{T. mentagrophytes} microconidia. In fact, n-valeric acid, n-isovaleric acid, isocaproic acid and isopentyl alcohol, at a concentration of 0.1 \% (w/v) suppressed microconidial germination induced by leucine. \textit{\delta}-Amino-n-valeric acid and N-benzoyl-amino-valerate at a concentration of 0.01 \% (w/v) somewhat stimulated leucine-induced germination. Isocaproic acid (4-methyl-valerate) was by far the most potent inhibitor among all the analogs tested. Penicillamine, a structural analog of valine, at concentration above 25 mM greatly stimulated the leucine-induced germination although it was not a good germination inducer by itself (Table 9).
<table>
<thead>
<tr>
<th>Compound</th>
<th>5 hr</th>
<th>8 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Carbamyl-L-leucine b</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Leucine ethyl ester HCl</td>
<td>35</td>
<td>55</td>
</tr>
<tr>
<td>Leucyl-leucine b</td>
<td>61</td>
<td>57</td>
</tr>
<tr>
<td>Leucyl-valine</td>
<td>68</td>
<td>82</td>
</tr>
<tr>
<td>Leucyl-tyrosine b</td>
<td>50</td>
<td>73</td>
</tr>
<tr>
<td>Valyl-leucine</td>
<td>37</td>
<td>65</td>
</tr>
<tr>
<td>Valyl-valine</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>Valyl-tyrosine</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Leucine</td>
<td>72</td>
<td>90</td>
</tr>
</tbody>
</table>

a Stock solutions (50 mM in H₂O) of each individual dipeptide were prepared and 0.5 ml samples of each peptide solution were added into small tubes containing 0.5 ml of microconidial suspension (1.1 X 10⁷ cells/ml in Na phosphate buffer, pH 6.0, 0.1 M). All tubes were incubated at 37 C on a rotary shaker.

b N-Carbamyl-L-leucine and leucyl-leucine were found to be less soluble in H₂O compared to the others. A milky solution was obtained in the case of leucyl-tyrosine.

c Germination percentages were estimated microscopically as previously described at the end of 3, 5 and 8 hr incubation time.
TABLE 9.

Effect of fatty acids and leucine analogs on leucine
induced germination a

<table>
<thead>
<tr>
<th>Compound</th>
<th>Activity b</th>
<th>0.1 %</th>
<th>0.01 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Valeric acid</td>
<td>I-2</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>(\delta)-Amino-N-valeric acid</td>
<td>I-1</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>N-Benzoyl-amino-valeric acid</td>
<td>S</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>n-Isovaleric acid d</td>
<td>I-2</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>N-Benzoyl-DL-leucine d</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Isopentyl alcohol d</td>
<td>I-1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>D-Penicillamine d</td>
<td>S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caproic acid ((C_6))</td>
<td>I-1</td>
<td>I-1</td>
<td></td>
</tr>
<tr>
<td>Isocaproic acid ((C_6)) d</td>
<td>I-2</td>
<td>I-2</td>
<td></td>
</tr>
<tr>
<td>Caprylic acid ((C_8))</td>
<td>I-1</td>
<td>I-1</td>
<td></td>
</tr>
<tr>
<td>Capric acid ((C_{10}))</td>
<td>I-1</td>
<td>I-1</td>
<td></td>
</tr>
<tr>
<td>Laurie acid ((C_{12}))</td>
<td>I-1</td>
<td>I-1</td>
<td></td>
</tr>
<tr>
<td>Myristic acid ((C_{14}))</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

a Solutions of each analog and fatty acid were prepared and added to the basic leucine-induced germination system at final concentrations indicated (0.1 %, 0.01 %, w/v).

b At the end of 8 hr incubation time, the germination percentages were estimated microscopically as described in the Materials and Methods section. The activity of individual compounds is indicated as: S: stimulatory, greater percentages of germination than the leucine-induced system (> 85 %); I-1: inhibitory, less germination percentages than the leucine-induced system; I-2: highly inhibitory (< 15 %), and O: no effect.

c Stimulation was observed only when 0.75 % was used.

d Structural formulae are shown in Fig. 14.
**Effect of ethylenediamine tetraacetic acid (EDTA) on germination.**

Preliminary studies carried out in the peptone system revealed that EDTA (0.3 mM) inhibited microconidial germination. As shown in Table 10, EDTA inhibited both the rate and the extent of germination of heat activated *T. mentagrophytes* microconidia.

**Effects of antifungal agents on germination.** In the presence of nystatin or amphotericin B, the microconidia were not able to germinate (Table 11). Moreover, the treated conidia became shriveled and developed large granules within the cytoplasm. Lysis of conidia was often observed after a prolonged period of incubation in the presence of these antifungal agents.

In contrast to this, griseofulvin and cycloheximide appeared to have no visible effect on conidial germination. However, branching, curling, swelling or the splitting of tips of elongated germ tubes was observed in the culture to which griseofulvin was added.

**Effect of protease inhibitor on microconidial germination.** Phenylmethylsulfonyl fluoride (PMSF), when incorporated into the germination system at a concentration as low as $10^{-6}$ M was found to inhibit the microconidial germination (Table 12). At a higher concentration ($10^{-3}$ M) complete suppression on germination took place. In contrast to this, PMSF exerted no inhibitory effect on the growth of germinated conidia.
## TABLE 10.

Effect of ethylene diamine tetraacetic acid (EDTA) on leucine-induced germination of *T. mentagrophytes* microconidia.

| EDTA concn (mM) in 25mM L-leucine | Germination (%) b |
|---|---|---|---|
| | 4 hr | 8 hr | 22 hr |
| 10.0 | 28 | 45 | 40 |
| 5.0 | 26 | 69 | 69 |
| 2.5 | 40 | 66 | 85 |
| 1.0 | 44 | 68 | 80 |
| 0.1 | 45 | 93 | 93 |
| 0. | 70 | 95 | 95 |

a In this experiment, heat-activated (45 C for 30 min) microconidia were employed in the basic germination system to which EDTA (0.1 mM to 10 mM) was added.

b Germination percentages were estimated (4, 8 and 22 hr) microscopically as described previously.
TABLE 11.

Effect of nystatin, amphotericin B, griseofulvin, and cycloheximide on germination of *T. mentagrophytes* microconidia

<table>
<thead>
<tr>
<th>Conc'n (µg/ml) of compounds tested</th>
<th>Germination (%) (3 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nystatin</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
</tr>
</tbody>
</table>

a All chemicals were pre-dissolved in 100 % dimethyl formamide (0.5 %) and then diluted to the desired concentrations with 1 % peptone. Microconidia were then inoculated in each preparation to give a final concentration of 5.0 X 10⁶ cells/ml. After 3 hr of incubation at 37 C on a rotary shaker, germination percentages were estimated microscopically as described in Methods. Dimethyl formamide (0.5 %) was found to have no inhibitory effect.

b The elongation of germ tubes occurred even in the presence of 100 µg/ml of these compounds.

c Not determined.
**TABLE 12.**

Effect of phenylmethysulfonyl fluoride (PMSF) on *T. mentagrophytes* microconidial germination\(^a\)

<table>
<thead>
<tr>
<th>PMSF concn (M) in 0.1% peptone</th>
<th>Germination(^b) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(10^{-3}) M</td>
<td>0</td>
</tr>
<tr>
<td>(10^{-4}) M</td>
<td>50</td>
</tr>
<tr>
<td>(10^{-5}) M</td>
<td>60</td>
</tr>
<tr>
<td>(10^{-6}) M</td>
<td>60</td>
</tr>
<tr>
<td>0.1% Peptone only</td>
<td>&gt; 95</td>
</tr>
<tr>
<td>1% Isopropanol only</td>
<td>&gt; 95</td>
</tr>
<tr>
<td>0.1% Isopropanol only</td>
<td>&gt; 95</td>
</tr>
</tbody>
</table>

\(^a\) Stock solution of PMSF (0.1 M) was prepared in 100% isopropanol. This was then diluted in peptone (0.1%) to give the desired final concentrations. Microconidia (5.0 X 10^6 cells/ml) were inoculated into each preparation and incubated at 37 C on a rotary shaker.

\(^b\) Germination percentages were estimated microscopically as described in Methods.
Effect of D-leucine on L-leucine-induced germination. D-leucine was found to induce germination of *T. mentagrophytes* microconidia as previously described (Table 4). However, the extent of germination was less than that obtained with L-leucine-induced germination (Table 4). D-leucine, when added to the basic germination system (L-leucine, 25 mM), was found to exhibit no inhibitory effect. Rather a slight stimulatory effect was observed when added at concentration levels of 10 mM and 25 mM (data not shown).
IV. DISCUSSION

The present study has demonstrated that a sequence of events similar to that which usually occur during bacterial spore germination (Fig. 13) also occurs during the germination of *T. mentagrophytes* microconidia. These events include the loss of refractility, resistance to heat, and resistance to stain by basic dyes, and an increase in respiratory activity (Fig. 7). At the same time the initiated microconidium increases somewhat in size (Table 3) and loses some of its dry weight and density (Table 3).

Although the protrusion of visible germ tube(s) from the dormant fungal spore wall is the most common criterion of germination encountered in the literature, there appears to be no consensus among mycologists as to the exact point at which a dormant spore is considered to be completely germinated (French, 1961; Manners and Hossain, 1963). According to Sussman (1966), germination is the first irreversible change which is recognizably different from the dormant organism, as judged by physiological or morphological criteria. The present findings that the *T. mentagrophytes* microconidia irreversibly lose refractility and heat resistance in the presence of L-leucine, without apparent germ tube emergence, seem to satisfy Sussman's definition for fungal spore germination, and appear to be a much preferred criterion for the germination of *T. mentagrophytes* microconidia than to the conventional criterion of germ tube protrusion.
Fig. 13. Stages involved in the transformation of bacterial and fungal spores into metabolically active cells.
It is a well known fact that certain single compounds, such as L-alanine and adenosine, trigger germination of certain bacterial spores (Powell and Hunter, 1955). To the author's knowledge, no single amino acid is known to induce germination of *T. mentagrophytes* microconidia.

It is generally accepted that the postgerminative outgrowth of spores is an energy-requiring process. The oxidation of endogenous reserves is believed to serve as the source of energy (Cochrane, 1966). An exogenous carbon source, such as glucose, is required when insufficient reserves are present (Cochrane, 1966; Manners, 1966). The ability of the phase-dark microconidia to develop short germ tubes in the phosphate buffer indicates that the endogenous reserves of these conidia are adequate for the limited subsequent vegetative outgrowth once dormancy is broken. This is in contrast to the bacterial spore system, in which there is not sufficient endogenous materials to support any post germinative development in the absence of exogenous carbon or nitrogen sources.

Lipids have been reported to be the major endogenous reserve in many fungal spores (Allen, 1965). Although both electron microscope (Fig. 3) and cytochemical studies (Fig. 2) indicate the presence of lipid bodies within the dormant *T. mentagrophytes* microconidia and their rapid disappearance during germination, the exact role of lipid in the germination process is still unknown. Undoubtedly, further extensive studies are needed before any definite role can be assigned to the microconidial lipid granules.
Some of the ultrastructural changes in cytoplasmic fine structures occurring during germination (Fig. 3 - Fig. 5) can be tentatively correlated with changes in the metabolic activity. The changes in shape and numbers of mitochondria (Fig. 4) could be related to the increase in respiratory activity (Fig. 7). The ruptures of the outer conidial wall are likely to be caused by "enzymatic degradation", as claimed by Marchant (1966). In this fungus, the new germ tube wall (VW) appears to originate from the innermost layer of the dormant conidial wall (IW, Fig. 5). Similar observations were also reported for Botrytis cinerea (Howker and Hendy, 1963), Aspergillus orzae (Tanaka, 1966), Aspergillus niger (Hawker, 1966). However, new germ tube walls of germinating Rhizopus nigricans (Hawker and Abbott, 1963) and Penicillium griseofulvum Dierckx (Fletcher, 1970) conidia appear to originate neither from the inner nor outer spore walls. It is now well established that the low metabolic activity of dormant spores is not due to their lack of enzyme systems in spores. Activation of enzymes already present in the spores during germination is well documented in the literature (Brambl and Van Etten, 1970).

The ranges of pH's (Fig. 9) and temperatures (Fig. 12) which permit germination of the microconidia imply that reaction involved in germination is enzymatic. The fact that the leucine-induced germination of T. mentagrophytes microconidia is not affected by cycloheximide (Table 11), an inhibitor of protein biosynthesis in eucaryotic cells, suggest that de novo synthesis of protein is not essential for this transition. This
interpretation is further supported by the evidence obtained previously in UV studies. It was found that the dormant microconidia could be made non-viable by large doses of UV irradiation without loss of their ability to germinate (Hashimoto et al., 1972). It therefore appears that enzymes involved in germination are likely to exist preformed, while those involved in postgerminative outgrowth must be synthesized de novo.

The loss of dry weight and specific gravity by microconidia during germination (Table 3) supports the degradative nature of germination process. Since this initial process of germination is primarily degradative in nature, the involvement of hydrolytic enzymes, such as proteases or glycosidases, seems probable. The observation that the addition of phenylmethylsulfonylfluoride, a selective protease inhibitor, completely inhibited the leucine-induced germination of T. mentagrophytes microconidia (Table 12) supports this speculation. The exact mechanism and the mode of action of the "germination enzymes" is not clear at the moment.

It is known that many fungus spores contain less water than do their corresponding vegetative cells (Allen, 1965). The fact that dormant T. mentagrophytes microconidia can be kept for a long time in water or phosphate buffer without swelling or germinating indicates the presence of some barriers that prevent the inflow of water in such spores. The thick wall of the dormant conidia (Fig. 3) is the most likely barrier and may also be responsible for the resistance to stain. The conidial walls are partially degraded during the germination and active metabolic activity appear to result following the imbibition of water.
Leucine seems to be the best compound for the initiation or triggering reaction of the \textit{T. mentagrophytes} microconidial germination. Although, at present, the exact mechanism of leucine-induced germination is still unknown, its involvement in active protein synthesis is questionable since germination was not at all inhibited by cycloheximide (Table 11).

The germination of some bacterial spores induced by certain L-amino acids is completely inhibited by its D-isomer (Ando, 1971). In \textit{T. mentagrophytes} microconidial germination, D-leucine is not only an active inducer but also stimulates the L-leucine-induced germination (Fig. 4). D-glucose was found to inhibit the L-leucine-induced germination (Hashimoto \textit{et al.}, 1972).

The presence of certain leucine aminopeptidases in \textit{T. mentagrophytes} have been reported (Male and Hobubar, 1968). This may account for the germination of the microconidia in the presence of the dipeptides containing L-leucine on their N terminus (Table 8), although the direct involvement of the dipeptides in the germination is also possible.

Short-chained fatty acids and leucine analogs were not only ineffective as germinants, but also were highly inhibitory to the leucine-induced germination (Table 9). Structures of some of these compounds tested are illustrated in Fig. 14. It appears that L-leucine with a modified amino group failed to induce germination and that analogs showing inhibitory effects had a similar terminal configuration. This suggests that the amino group in combination with the terminal isopropyl group might be important as effective germinants.
L-LEUCINE

N-CARBAMYL-DL-LEUCINE

N-BENZOYL-L-LEUCINE

ISOCAPROIC ACID

L-VALINE

N-ISOVALERIC ACID

ISOPENTYL ALCOHOL

PENICILLAMINE

Fig. 14. Structures of selected leucine analogs
Various factors such as, leucine concentrations (Table 4), size and age of the inoculum (Table 6), pH's (Fig. 9), temperatures (Fig. 12), and heat (Fig. 8) were all shown to have marked influence on the leucine-induced germination of the *T. mentagrophytes* microconidia. This suggests that the triggering reaction of germination could be enzymatic rather than physical. The activation of lytic enzymes by a L-leucine complex at a stereospecific binding site in the conidia is probable. However, further studies of the metabolism of leucine during germination should be undertaken before any conclusion is drawn.

The pretreatment of microconidia with sublethal doses of heat (45°C, 30 min) seems to have the most pronounced effect on the leucine-induced germination (Fig. 10). Heat shock is thought to influence the germination by altering the properties of the germination enzymes, changing the permeability, or stimulating the reaction(s) involved in germination (Sussman, 1966). However, the exact site in the microconidia activated by such processes is unknown.

On the basis of the observations made in the present study, a schematic model of the process of germination is proposed in Fig. 15. It is postulated that during this transformation from a dormant microconidium into a vegetative cell, two main reactions are considered essential: the triggering reaction and the enzymatic breaching of the dormant conidial wall. L-Leucine might be responsible for the triggering reaction during which certain hydrolyzing enzyme(s) are activated or released. These enzymes could then breach the conidial wall, causing
water influx and resulting in the swollen, phase dark and metabolic active cell, thus drastically altering their physiological and morphological properties from those of the dormant microconidium.
Fig. 15. Diagramatic illustration of the proposed mechanism of germination of *T. mentagrophytes* microconidia.
V. SUMMARY

The microconidia of *T. mentagrophytes*, a fungus responsible for superficial human dermatomycoses, undergo synchronous germination via phase darkening, swelling, and germ tube formation within 2 hr in peptone (0.5 %) at 37 C. During germination, the fungal spores were shown to become phase dark prior to germ tube development. Several properties inherent to dormant microconidia, such as resistance to heat and stain and the inability to use glucose, were observed to change during this transition. A reduction in conidial dry weight and specific gravity were also noted. A search for the effective component which triggered germination of *T. mentagrophytes* microconidia resulted in the identification of 7 amino acids (leucine, isoleucine, alanine, methionine, valine, tryptophan and glycine) among which L-leucine proved to be the most effective germiant. Carbohydrates, nucleosides, nucleotides, salts, vitamins, or other compounds tested, did not induce germination of the fungus conidia, nor did these have any effect on the L-leucine-induced germination. D-leucine, and a few dipeptides containing L-leucine as the N-terminal amino acid, however, were effective as germinants. Several short-chained fatty acids and selected leucine analogs were, in general, inhibitory to microconidial germination induced by L-leucine.

The concentration of L-leucine, the age and size of the inoculum, the pH and the temperature significantly affected the L-leucine-induced germination of *T. mentagrophytes*. Most rapid germination was obtained
when microconidia (5.0 x 10^6 cells/ml) were incubated in the presence of L-leucine (25 mM) at 37 °C with a pH range between 6.5 to 7. The germination rate was enhanced significantly when the conidial suspension was preheated at 45 °C for 30 min. Anaerobic conditions, and the presence or absence of carbon dioxide did not significantly affect microconidial germination. The antifungal antibiotics amphotericin B and nystatin and phenylmethylsulfonyl fluoride, a protease inhibitor, inhibited germination completely. In contrast to this, griseofulvin and cycloheximide appeared to have no visible effects on the microconidial germination. Electron microscopy revealed that the major ultrastructural changes of the microconidia observed during germination included the disappearance and fragmentation of large lipid bodies, the changes in mitochondrial shape, the development of extensive membranous structures and the rupturing of the outer conidial wall.

The need for the establishment of a more rigorous definition of germination of fungus spores was emphasized. Phase darkening and loss of heat resistance appear to be suitable parameters for the assessment of germination of *T. mentagrophytes*. The possible mechanism of L-leucine as a germination trigger in the early phase of germination of *T. mentagrophytes* microconidia was discussed.
VI. REFERENCES


Ando, Y. 1971. The germination requirements of spores of Clostridium botulinum Type E. Japan J. Microbiol. 15: 515-525.


APPROVAL SHEET

The thesis submitted by Christine Da-Ruh Wu has been read and approved by the members of the Advisory Committee listed below.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

December 22, 1972
Date

Signature of Advisory Committee Director

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