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FACTORS AFFECTING GERM TUBE FORMATION IN *CANDIDA ALBICANS*

by

Walter A. Tatarowicz

A Thesis Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirement for the Degree of Master of Science

October

1985

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VITA

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He is the co-author of the following publications:

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Thompson, K.D., J.P. O'Keefe, and W.A. Tatarowicz. 1983. In vitro activity of the quinilone WIN 49375 against 198 clinical isolates including aminoglycoside resistant *Pseudomonas aeruginosa*. Abstr. Annu. Meet. Interscience Conf. Antimicrob. Agents Chemother. 376, p. 151.

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
arg	L-arginine
BME	Basal Medium Eagle
^{o}C	degree Celcius
cfu/ml	colony forming unit per milliliter
cys	L-cysteine
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
^{o}F	degree Fahrenheit
Fig	figure
g	grams
x g	times force of gravity
GlcNAc	N-acetyl-D-glucosamine
gln	L-glutamine
gly	L-glycine
g.t	germ tube
h	hours
his	L-histidine
ile	L-isoleucine
l	liters
leu	L-leucine
lys	L-lysine
M	molar

ManNAc.................. N-acetyl-D-mannosamine

met..................... L-methionine

min..................... minutes

ml...................... milliliter

mM...................... millimolar

%....................... percent

phe..................... L-phenylalanine

pro..................... L-proline

RNA..................... ribonucleic acid

SDA..................... Sabouraud Dextrose Agar

SDB..................... Sabouraud Dextrose Broth

ser..................... L-serine

TC199................... Tissue Culture Medium 199

thr..................... L-threonine

TMN..................... Tris-maleate-NaOH

tyr..................... L-tyrosine

μm...................... micrometer

val..................... L-valine

v/v..................... percent by volume

INTRODUCTION

The purposes of this thesis research are (1) the identification and characterization of a component(s) in Dulbecco's Modified Eagle's Medium (DMEM) synthetic tissue culture medium that can singly induce germ tube formation in *Candida albicans*, and (2) the examination of environmental and nutritional factors affecting the induction of germ tube formation of *C. albicans* ATCC 58716.

Initially, the general aspects of germ tube formation will be studied using a commercially available, synthetic medium which promotes complete germ tube formation. Environmental conditions which promote optimal germ tube formation will be determined. Once the environmental parameters have been established, attempts will be made to identify (a) component(s) in the medium that is(are) responsible for for germ tube inductive activity of the tissue culture medium. Since the exact formulation of the synthetic medium is known and all components are readily available, elimination and reconstitution experiments should lead to the development of a simplified minimal medium which, hopefully, contains only a single inducer. Once such (a) component(s) is(are) identified, the induction of germ tube formation by the compound(s) will be physiologically characterized. Ultimately, the data presented here will

be compared and contrasted to work from other investigators, which may lead to a better understanding of induction of germ tube formation in the dimorphic fungus C. albicans.

The phenomenon of fungal dimorphism has intrigued researchers in the medical and scientific professions for many years. It is thought that the elucidation of the basic mechanisms of dimorphism would lead to a better understanding of pathogenic mechanisms of certain fungal infections and to a better understanding of differentiation and growth control in eucaryotic organisms. Though most investigators have approached dimorphism by examining the effect of physical, chemical, and nutritional factors on the inducement or repression of dimorphism, it is now believed that the basic understanding of dimorphism requires integrated experimental approaches involving cytology, physiology, biochemistry, and genetics.

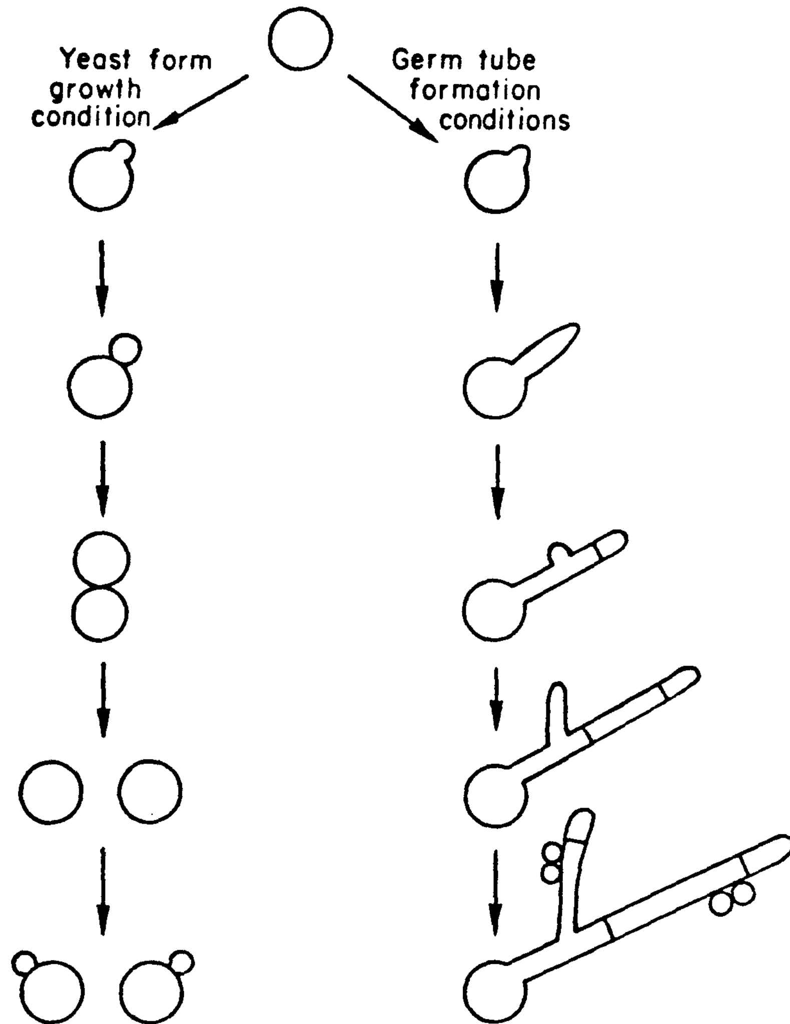
In nature, some pathogenic fungi exist in two states: saprophytic or parasitic. Morphologically, many of such pathogenic fungi display dimorphism, meaning that they assume two distinct morphologies depending on their saprophytic or parasitic phase of growth. In more general terms, dimorphism describes environmentally controlled interconversions of the two morphological forms. Some important dimorphic fungi include the pathogens Blastomyces dermatiditis, Histoplasma capsulatum, Paracoccidi-

oides brasiliensis, Coccidioides immitis, and Candida albicans, as well as the non-pathogenic Mucor rouxii.

Candida albicans is a dimorphic fungus capable of existing in a yeast (Y) form or a hyphal (H) form. The yeast cell, the unicellular spherical or oval form of C. albicans, is distinguished by a specific mode of cell multiplication known as budding (Fig. 1). This process involves the accumulation of new cellular material at a selected site on the parent yeast cell surface. This bud then enlarges, nuclear division takes place followed by the migration of one of the chromatids into the bud, and a narrow septum is constructed between the parent and daughter cells. A hypha is a long tube or filament comprised of single or multiple cell units divided by septa. Hyphae can arise as branches of existing hyphae or by induction of tubular growth from a parent yeast cell (Fig. 1). The parent cell lays down new cellular material at a selected site on the parent yeast cell surface. The "germ tube" then grows continuously by extension. Mitotic cell division occurs within the extending germ tube and a septum is formed within the germ tube. Once the elongating cell becomes multicellular, it is then generally called a hypha. Under certain conditions, secondary yeast cell formation eventually occurs along the hyphal wall posterior to septae.

Ecologically, C. albicans usually exists as a member

Fig. 1. Morphology of C. albicans. C. albicans yeast cell replication occurs by bud formation under yeast form growth conditions or by elongation during germ tube formation conditions.


Yeast form
growth
condition
Germ tube
formation
conditions

of the normal human microbial flora. But in patients with cancer, diabetes, or immunodeficiences, or in patients undergoing broad spectrum antibiotic therapy or immunosuppresive therapy, _C. albicans_ is capable of causing a life-threatening disease (Edward et al., 1978; Lehrer and Cline, 1971; Rippon, 1974). It has been suggested that a change in the cellular morphology of _C. albicans_, from Y to H form, may play an important role in the virulence of the organism (Louria et al., 1962; Martin et al., 1984). Evidence in most reports is based on the fact that the hyphal form is generally seen in smears, scrapings, or histological sections from _C. albicans_ lesions, and that hyphal formation occurs within hours of inoculation of yeast cells in tissues of experimental animals (Odds, 1979). Yet, Klotz et al. (1983) demonstrated that the _C. albicans_ yeast form was capable of adherence as well as penetration into porcine endothelium. They suggested that, though the yeast form may initiate the invasive process, it is likely that germ tube formation potentiates the invasive process. There is conflicting evidence, though, as to the ability of the hyphal form to escape killing and elimination by the body's cellular immune system (Diamond et al., 1978; Lehrer and Cline, 1971; Schewitz and Martin, 1979; Stanley and Hurley, 1969). Nevertheless, study of the dimorphic nature of _C. albicans_ may shed new light on the pathogenicity of this fungus and, quite possibly, generate the development

of superior antifungal treatment.

The study of the dimorphic nature of *C. albicans* can be traced as far back as 1887. Upon microscopic observation of yeast and hyphal forms of *C. albicans*, Audrey (1887) posulated that the different morphological forms arose from one organism, and that the predominating form depended on the growth medium. From the early 1900's to the late 1960's, numerous papers and comprehensive reviews (Cutler and Hazen, 1983; Odds, 1979; San-Blas and San-Blas, 1983; Szaniszlo et al., 1983) had been published citing the environmental and nutritional factors which affected the growth and shape of *C. albicans*. Yet, in spite of the large number of studies investigating *C. albicans* dimorphism, the resultant data are inconsistent and often contradictory. For example, the responses to specific inducers or to identical incubation conditions varied from laboratory to laboratory. The explanation for this is thought to be threefold. First, strain variation has been a well-documented phenomenon (Dabrowa et al., 1976; Hrmova and Drobnica, 1981; Manning and Mitchell, 1980a; Torres-Bauza and Riggsby, 1980). Even within the setting of a single laboratory under identical experimental conditions, a compound may induce certain strains and have very little effect on other strains. Secondly, most investigators used different media and different culture conditions, making comparisons between the studies difficult. Final-

ly, there have been differences in the nomenclature of the events and growth forms during _C. albicans_ dimorphism. Depending on the investigator, synonyms for the yeast form include blastospore and blastoconidia. Likewise, germ tube, pseudohypha, mycelium, and filament are terms synonymous with hypha. Depending on the investigator, hyphal induction may also be known as germ tube, hyphal, or mycelial formation, as well as filamentation or germination. It is possible that a difference in nomenclature between two groups might have actually represented the same morphological type, and vice versa. For these reasons, summation of data leaves many of the basic questions about dimorphism unanswered.

In recent yeast, some attempts have been made to study _C. albicans_ growth and dimorphism using defined liquid culture media and fairly standard culture conditions.

Various synthetic tissue culture media have been used to induce hyphal growth. Modified Eagle's Medium supplemented with trace ions, glucose, and glycine supported greater than 95% hyphal growth at 40°C for nearly 72 h incubation (Schwartz and Larsh, 1980). When the cultures were incubated at 20°C, yeast growth occurred. Hazen and Cutler (1979) used another tissue culture medium, TC199, to demonstrate 86% germ tube formation at 37°C after 3 h incubation in 5% CO_2. Dabrowa et al., (1970) also observed 95% germ tube formation in TC199 after 3 h

incubation at 37°C in air.

Bovine seminal plasma was shown to contain a peptide and a glycopeptide which together induced germ tube formation in C. albicans at 37°C (Chattaway et al., 1980). Both peptides contained appreciable amounts of aspartate and glutamate. The glycopeptide also contained threonine, lysine, histidine, and arginine, while the peptide also contained proline. Analysis of the glycopeptide indicated the presence of N-acetylglucosamine (21%).

Mardon et al. (1969) described one of the first synthetic media which supported C. albicans pseudohyphal growth. Testing only one strain, they found that their glucose-salts-biotin medium, supplemented with either L-methionine, L-phenylalanine, or L-tyrosine as the nitrogen source, induced a high percentage of hyphal growth. They later showed similar results in the same medium where L-alpha-n-butyric acid had been substituted for the amino acids (Mardon et al., 1971). Lee et al., (1975) developed a mixture of several amino acids, biotin, inorganic salts, and glucose that reproducibly induced hyphal formation in C. albicans. This medium has been used by numerous investigators (Chaffin and Sogin, 1976; Manning and Mitchell, 1980a; Mitchell and Soll, 1979; Torres-Bauza and Riggsby, 1980). Most recently, it has become possible to induce germ tube formation in a simple medium containing a single amino acid. Proline alone (Dabrowa et al., 1976), with

the addition of biotin and glucose (Land et al., 1975), or supplemented with biotin, glucose, and NH_4Cl (Soll and Bedell, 1978) has been shown to induce substantial hyphal formation. Similarly, glutamine plus glucose (Shepherd et al., 1980) and glutamine plus biotin (Nishioka and Silva-Hunter, 1974) both supported substantial hyphal formation. N-acetylglucosamine (GlcNAc), a chitin precursor, is another hyphal inducing compound which has been used by several groups (Chiew et al., 1980; Gopal et al., 1982; Shepherd et al., 1980; Simonetti et al., 1974). Also, three groups reported germ tube formation in media containing only $(NH_4)_2SO_4$, salts, glucose, and/or vitamins (Hrmova and Drobnica, 1981; Manning and Mitchell, 1980a; Yamaguchi, 1975). It is worth noting that germ tube formation in all these media was achieved by incubation at 37°C.

It is generally agreed that incubation at temperatures greater than 35°C is usually necessary for promotion of candidal hyphal formation in the appropriated inducing media (Chattaway et al., 1976; Dabrowa et al., 1970; Evans et al., 1975; Lee et al., 1975; Mattia and Cassone, 1979; Nishioka and Silva-Hunter, 1974; Simonetti et al., 1974). However, Hrmova and Drobnica (1981) observed substantial germ tube formation at incubation temperatures as low as 28°C, but with much strain variation. Bedell and Soll (1979) isolated a mutagenized strain of _C. albicans_ which

was capable of approximately 60% germ tube formation in Lee's medium at 25°C, but only after an extended incubation time of 40 h.

Several studies have examined the effect of pH on germ tube formation in C. albicans. Evans et al. (1975) evaluated germ tube formation over a pH range of pH 3 to pH 9 in a broth composed of 1% peptone and 0.2% glucose. No mention was made as to the method of pH adjustment. Substantial germ tube formation occurred between pH 5 and pH 9, with complete hyphal formation only at pH 7.4. Testing only two pH values, Mardon et al. (1971) observed 5-20% more germ tube formation at pH 6.95 than at pH 5.7 in their L-alpha-amino-n-butyric acid induction medium. Mitchell and Soll (1979) found that Lee's medium induced germ tube formation at pH 6.5, while at pH 4.5, only budding occurred. Of five different media tested for hyphal induction, Evans et al. (1974) observed highest mycelial yields in complex media with a slightly alkaline pH.

Inoculum concentration has been shown to be a critical factor in germ tube formation. Most studies have demonstrated a decrease in germ tube formation at inoculum concentrations greater that 10^7 cfu/ml (Evans et al., 1975; Muerkoester et al., 1979). Dabrowa et al. (1976) and Mardon et al. (1969) demonstrated, in their two systems, a decrease in germ tube formation at inoculum concentrations greater than 10^6 cfu/ml.

It was thought that the growth phase of the inoculum played an important role in the ability of the yeast cells to form germ tubes. Until 1979, most investigators believed that only stationary phase cells were capable of producing germ tubes when transferred to an induction medium (Chaffin and Sogin, 1976; Evans et al., 1975; Mitchell and Soll, 1979). Yet, Mattia and Cassone (1979) and Dabrowa and Howard (1981) recently demonstrated that exponential phase cells could also be induced to form germ tubes when they were placed in rich media, such as serum. Soll and Herman (1983) took this one step further by showing that exponential phase cells which were starved in a buffered salts solution for 20 to 40 minutes could be induced to form germ tubes in Lee's amino acid medium. Aherns et al. (1983) also noted germ tube production in Lee's medium by one strain of _C. albicans_ regardless of growth phase of the inoculum. Exponential phase cells displayed more rapid and more synchronous germ tube production when compared to stationary phase cells.

The use of chemically defined culture media as well as standard culture conditions (incubation temperature of 37°C, inoculum concentration of less than 10^7 cfu/ml) have also facilitated the study of ion effects on hyphal formation.

Zinc has been shown to both suppress and enhance hyphal formation. In their salts-glucose-biotin-

$(NH_4)_2SO_4$ germination system, Yamaguchi et al. (1975) recorded decreased germ tube formation upon the addition of zinc. Bedell and Soll (1979) observed that micromolar concentrations of zinc did not inhibit germ tube formation and that, if zinc was present in the growth medium of the original yeast culture, germ tube formation time was substantially decreased. Ross (1982) noted that though zinc did not affect germination, it did suppress subsequent extension of germ tubes to form mycelia. The yeast cells that were present did bud normally, indicating that growth and multiplication were not suppressed by zinc. Most recently, Anderson and Soll (1984) observed that yeast cells which had accumulated in stationary phase due to zinc depletion formed germ tubes approximately 40 min later than yeast cells which had accumulated in stationary phase due to depletion of some other ion or nutrient. They theorized that the zinc-depleted stationary phase yeast cells may have had to reaccumulate zinc in order to resume protein synthesis.

Recently, Walker et al. (1984) reported that GlcNAc induced germ tube formation occurred only in the presence of manganese (40% germ tube formation) or magnesium (80% germ tube formation). Addition of two other divalent cations, calcium or zinc, to the GlcNAc medium did not stimulate germ tube formation. It was also shown that the addition of magnesium enhanced $[^{14}C]$GlcNAc incorporation in

hyphal cells.

There is contradictory evidence concerning the role of phosphate in hyphal formation. Elimination of phosphate from the minimal amino acid/salts mixture of Chattaway substantially decreased the amount of germ tube formation (Chattaway et al., 1976). High concentratons of phosphate (greater than 1 mM) in a proline medium depressed filamentation (Land et al., 1975). Similarly, Simonetti et al. (1974) and Shepherd et al. (1980) found that germ tube formation in their GlcNAc medium did not occur when the inducer was diluted in phosphate buffer rather than imidazole buffer.

Certain studies suggest that morphological conversion of C. albicans may be controlled by substances called autoinhibitors, self-inhibitors, or autoantibiotics. These substances accumulate in the culture medium during incubation and inhibit or significantly retard the growth and development of the organisms producing them. Lingappa et al. (1969) isolated two autoantibiotics produced by C. albicans. Both phenethyl alcohol and tryptophol were shown to be accumulated in culture medium, and addition of either to fresh medium inhibited the growth of C. albicans. Addition of 0.2-0.3% phenethyl alcohol to serum also resulted in the suppression of germ tube formation, and led to the subsequent development of budding yeast cells (Hendry, 1972). Hazen and Cutler (1979) reported a morphogenic

regulatory substance (MARS) which was capable of influencing germination. Yet, when compared to standard gas chromatographs of phenethyl alcohol and tryptophol, no such peaks were detected in their hyphal induction medium either before or after incubation of C. albicans. Shepherd et al. (1980) indicated that phenethyl alcohol was effective in preventing germ tube formation.

It has been proposed that cysteine might promote yeast cell formation rather than hyphal formation. In 1954, Nickerson (1954) postulated that the enzymatic transfer of reducing power generated within the mitochandria to the yeast cell wall via a disulfhydral reductase controlled morphogenesis. He suggested that a medium rich in cysteine (and thus reduced) would promote yeast form growth. Simonetti et al. (1974) reported that addition of 0.1 mM cysteine to their GlcNAc germination system completely inhibited germination. Yet, Nishioka and Silva-Hunter (1974) presented conflicting results, showing that cysteine (0.1-10 mM) failed to suppress mycelial development in their glutamine medium. Wain et al. (1975) noted suppression of hyphal growth upon the addition of 10 mM cysteine to plasma. However, there was no suppression of initiation of hyphal formation. Also, the regulatory factor (MARS) described by Hazen and Cutler (1979) was shown not to be cysteine.

Biochemical differences between yeast and hyphal cells

of *C. albicans* have been investigated by a number of workers. Dabrowa et al. (1970) observed that, within the first two hours of hyphal growth in TC199, protein and RNA increased 49% and 94%, respectively, while there was no change in the amount of DNA. Similarly, Shepherd et al. (1980) observed a twofold increase in RNA after 4 h of hyphal growth while the amount of DNA remained unchanged. It was suggested that DNA replication was not necessary for induction of hyphal formation. Wain et al. (1976) described exponential increases in RNA and stepwise exponential increases in DNA content during hyphal growth. Using a nuclear staining technique, they were able to show that hyphal nuclear division occurred after hyphal synthesis, again suggesting that DNA synthesis was not necessary for hyphal induction. The intracellular level of cyclic AMP has been reported to increase during hyphal growth (Chattaway et al, 1981; Niimi et al., 1980), though Sullivan et al. (1983) detected no change in the level.

There is evidence that even certain proteins are present only in one or the other form. Manning and Mitchell (1980b) compared cytoplasmic proteins from two different strains of *C. albicans*. They found that a number of proteins separated by polyacrylamide gel electrophoresis differed in incorporation intensity between budding and hyphal forming cells. Electrophoretic analysis of water soluble proteins from budding and hyphal cells in another study

(Dabrowa et al., 1970) revealed six common bands, four bands specific for budding cells, and a single protein band unique for hyphal cells. Using another technique, Dabrowa and Howard (1984) examined the heat shock proteins (those synthesized after an increase in incubation temperature) and heat stroke proteins (those repressed after an increase in incubation temperature). Upon a temperature shift from 25°C to 37°C, yeast cells growing in Lee's medium were induced to form hyphae. Cellular protein analysis revealed the appearance of five heat shock proteins in the hyphal form as well as two heat stroke proteins in the yeast form. The same seven proteins were also found in a non-germinating variant of the parent strain.

Indirect fluorescent antibody staining techniques have demonstrated the presence of specific mycelial antigens on the mycelial portion of the hyphal cell (Smail and Jones, 1984).

The cell wall compositions of the yeast and hyphal forms of *C. albicans* are qualitatively similar, but quantitatively distinct. An increased amount of chitin, a major cell wall component in *C. albicans*, has been associated with the hyphal form. Chattaway et al. (1968) noted three times as much chitin in the hyphal cell wall as compared to the yeast cell wall while Sullivan et al. (1983) noted a fourfold increase. Both forms appear to have similar concentrations of lipid, protein, and total carbohydrate.

The mannan content of the yeast and hyphal walls is similar 20-23%), whereas the quantity of alkali-soluble glucan is slightly greater in the hyphal cell wall (Sullivan et al., 1983).

A review of dimorphism in the other pathogenic fungi reveals many similarities to dimorphism in C. albicans.

Initially, it is worth noting that in contrast to C. albicans, the parasitic stage of the other pathogenic fungi is the yeast form, while the hyphal form or spore form is the saprophytic stage. However, in these other fungi, as well as in C. albicans, conversion to the parasitic phase, regardless of the final form, occurs upon a temperature shift from 25°C to 37°C, or from room temperature to human body temperature. This phenomenon is generally known as thermal dimorphism.

Besides incubation temperature, nutritional and environmental factors have been shown to be a critical determinant in dimorphism in the other pathogenic fungi. For example, it is thought that, in Histoplasma capsulatum, cystine, cysteine, serine, or unknown organic sulfur molecules with reduced sulfur groups are responsible for conversion to the yeast form (Scherr, 1957). Transport of cystine is regulated by a permease which is active only at 37°C. In this case, temperature may regulate the synthesis or function of a key enzyme(s) that ultimately determines phase morphology. Manganese is critical for con-

version to, and maintenance of, the hyphal form of *Phialophora verrucosa*, a chromomycotic fungus (Reiss and Nickerson, 1971). The ion may function as a cofactor in cell wall biosynthesis. Cell density has been shown to be a critical determinant in conversion in *Exophiala werneckii* (Hardcastle and Szaniszlo, 1974), the causative agent of tinea nigria, and in *Coccicioides immitis* (Lones and Peacock, 1960). And, as most notable as in *C. albicans*, strain variation is a common occurence in all the pathogenic fungi (Szaniszlo et al., 1983).

In most of the pathogenic dimorphic fungi, differences in cell wall composition of the yeast and hyphal forms have been quantitative rather than qualitative (Szaniszlo et al., 1983). Minor differences in soluble proteins of yeast and hyphal forms have been noted in *Blastomcyes dermatiditis* (Roy and Landau, 1972).

MATERIALS AND METHODS

Organism. Candida albicans strain LUMC101 serotype A, isolated from a patient with disseminated candidiasis at Loyola University Medical Center, was used throughout the study. This strain has recently been deposited in the American Type Culture Collection (ATCC) and has been designated ATCC strain 58716.

Maintenance of the Organism. Stock cultures of the organism were maintained at room temperature on Sabouraud Dextrose Agar (SDA; Difco Laboratories, Detroit, MI) and transferred onto fresh SDA weekly. Working cultures were transferred onto fresh SDA daily and incubated at 37°C.

Preparation of Standard Yeast Cell Inoculum. Isolated colonies from a 24 h SDA culture were suspended in either 0.85% saline or 0.05 M Tris(hydroxymethyl)aminomethane-maleate-NaOH buffer (Tris-maleate or TMN buffer) pH 7.0 (Sigma Chemical Co., St. Louis, MO). The cells were then washed by centrifugation three times with TMN buffer and finally resuspended in TMN buffer. Yeast cells were then diluted in TMN buffer to a reading of 100 Klett units (filter #54; Klett-Summerson Photoelectric Colorimeter, Model 800-3, Klett Mfg. Co., New York, NY) which corresponded to a concentration of approximately 1.3×10^7 cfu/ml. The suspension was diluted tenfold with TMN buffer to a final

working concentration of 1.3 x 10^6 cfu/ml. At this point, the suspension was examined microscopically to obtain a morphological profile of the the inoculum. Suspensions containing any hyphal cells were discarded. Any suspension containing more than 20% budded cells was also discarded. Preparation of the standard yeast cell inoculum was done under sterile conditions.

Germ Tube Induction Media. Table 2 lists the commercially available, dehydrated media, serum, or tissue culture media which were tested for their ability to induce germ tube formation. All dehydrated media were prepared according to the manufacturer's instructions.

BME Amino Acid Solution (GIBCO) and BME Vitamin Solution (GIBCO) were supplied as 50x and 100x solutions, respectively. For use in our germ tube induction system, they were first diluted in sterile, distilled water to give a working dilution concentration of 10x.

Arginine (arg) and glutamine (gln) media were prepared from 10x stock solutions of each of the following components: salts, $CaCl_2$, and either arg or gln. The composition and concentration of each stock solution are described in Table 1. In certain experiments, when various other components were included in the media, they were also prepared as 10x stock solutions.

DMEM (GIBCO) was supplied as low glucose (5.55 mM), with L-glutamine (4.00 mM) and sodium pyruvate (1.17 mM).

Table 1. Composition of stock (10X) solutions.

Stock	Components	Concentration (mM)
Salts[a]	KCl	53.7
	NaCl	1095.1
	$MgSO_4 \cdot 7H_2O$	8.1
	$NaHCO_3$	440.5
	$NaH_2PO_4 \cdot H_2O$	9.1
	$FeSO_4 \cdot 7H_2O$	8.1
$CaCl_2$[a]	$CaCl_2$	18.0
Arg[b]	L-arginine	4.0
Gln[b]	L-glutamine	40.0
Glucose	Glucose	55.5

[a]Prepared in distilled deionized water.

[b]Prepared in TMN buffer.

Unless otherwise noted, all amino acids and sugars used in this study were L-amino acids and D-sugars.

Germ Tube Induction System. Germ tube induction in the commercially available, dehydrated media was tested by diluting the standard yeast cell inoculum tenfold with the medium.

Sample tubes of arg of gln media consisted of 0.2 ml of each stock solution, 0.2 ml of the previously described standard yeast cell inoculum, and 1.2 ml of TMN buffer. When other components were included in the media, they were also added as 0.2 ml from a 10x stock solution. The amount of TMN buffer (1x) was adjusted accordingly.

Unless otherwise stated, all inoculated tubes were incubated at a 30° angle at $37^{o}C$ for 4 h. At various intervals during the incubation period, samples were removed and examined microscopically. A minimum of one hundred cells were observed and the result was expressed as the percentage of cells forming germ tubes or hyphae. Germ tube lengths were measured in those cells which formed germ tubes and average germ tube length was calculated using these values. Cells which did not form germ tubes were not included in the calculation of average germ tube length. In some instances when samples could not be examined immediately, 0.1 ml of 10% formalin was added to the tubes to fix the cells.

All light microscopy of yeast and hyphal cells was

performed with a Nikon light microscope (model L-Ke) fitted with phase contrast objectives (40x and 100x). Germ tube lengths were measured using a calibrated micrometer placed in the eye lens. Photomicrographs were taken with a Nikon M-35S camera equipped with an automatic exposure system attached to the microscope using Plus-X Pan film (Eastman Kodak Co., Rochester, NY).

Preincubation of Inoculum in TMN Buffer. Glass tubes containing 10 ml of standard yeast cell inoculum were incubated at 37°C, 25°C, 20°C, 15°C, or 6°C for various lengths of time. For germ tube induction studies following this preincubation, cells were inoculated directly into the appropriate medium using standard inoculation conditions. In one experiment, 10 ml of standard yeast cell inoculum was incubated at 20°C for 12 h. The cells were then washed with TMN buffer by Millipore filtration (0.45 um pore size Metricel filter, Gelman Sciences Inc., Ann Arbor, MI), and resuspended in fresh TMN buffer prior to inoculation into the induction medium.

Growth of C. albicans Yeast in Sabouraud Dextrose Broth. A sidearm flask containing Sabouraud Dextrose Broth (SDB; Difco Laboratories, Detroit, MI) supplemented with 2% dextrose (Sigma Chemical Co., St. Louis, MO) was inoculated with standard yeast cell inoculum to yield a final concentration of 1.3×10^5 cfu/ml. The flask was then incubated at 37°C with shaking, Klett readings were

taken at appropriate intervals, and samples were removed accordingly for further testing.

Germ Tube Commitment Time. Sterile, clean glass coverslips (American Scientific Products, McGaw Park, IL) in plastic Petri dishes (American Scientific Products, McGaw Park, IL) were overlaid with a suspension of the standard yeast cell inoculum. Cells were allowed to settle and adhere to the coverslips for 20 min at 37°C. At that point, a control coverslip was removed and examined microscopically for any germ tube formation. Test coverslips were then washed once in TMN buffer to remove any unattached cells, placed in clean Petri dishes, and overlaid with DMEM. Groups of coverslips in DMEM were then incubated at 37°C or 4°C. Three coverslips from each group were removed at various intervals. The first coverslip was assessed for germ tube formation. The second coverslip was washed once in TMN buffer and placed in a clean Petri dish containing fresh TMN buffer. This tested for the possibility of medium carryover. The remaining coverslip was washed five times in TMN buffer and placed in another Petri dish containing fresh TMN buffer. These two remaining coverslips were then incubated at 37°C for 3 h and examined for germ tube formation.

Nuclear Staining. Yeast cells and germ tubes were fixed by adding two volumes of absolute ethanol to one volume of cells. These suspensions were incubated at room

temperature for 30 min. The ethanol-fixed cells were then diluted with twenty volumes of water to one volume of cells and stored at room temperature.

Prior to staining, the fixed cells were washed once with distilled water by Millipore filtration (0.45 µm pore size Metricel filter, Gelman Sciences Inc., Ann Arbor, MI). The following staining steps were done in a darkened room. The washed cells were resuspended in a small volume of 4',6-diamidino-2-phenylindole (DAPI; Sigma Chemical Co., St. Louis, MO; final concentration, 0.2 µg/ml) and incubated for 5 min at room temperature. The stained cells were washed once in distilled water by Millipore filtration to wash out the DAPI and finally resuspended in glycerol:water (1:1, vol/vol). Samples were placed on clean glass slides, coverslips were placed on top of the samples, and the edges were sealed with clear plastic fingernail polish. The slides were then stored at 4°C in the dark until microscopy was performed. Microscopy was performed using a Leitz fluorescence microscope (Dialux, 100x objective).

<u>Source of Chemicals</u>. Calcium chloride, citric acid, ferric sulfate, formalin, glycerol, hydrochloric acid, magnesium sulfate, and dihydrogen sodium phosphate were all obtained from J.T. Baker Chemical Co., Phillipsburg, NJ. Monobasic sodium phosphate was obtained from Fisher Scientific Co., Pittsburgh, PA. D-glucose, glacial acetic acid, maleic acid, potassium chloride, sodium bicarbonate,

and sodium hydroxide were obtained from Mallinkcrodt, Paris, KT. Sodium chloride was obtained from MCB, Norwood, OH. Amino acids, dihydrogen potassium phosphate, piperazine, sodium acetate, Trizma (Tris) acid, Trizma (Tris) base, and Trizma-HCl were obtained from Sigma Chemical Co., St. Louis, MO. HI-WO tissue culture medium was obtained from ISI (International Serum Institute, St. Louis, MO).

Statistical Analyses. Differences in amounts of germ tube formation were analyzed using the paired t-test.

RESULTS

Germ Tube Formation in Various Complex and Synthetic Media. In order to be able to study the factors involved in germ tube induction in *C. albicans*, we first had to find a medium which could induce complete germ tube formation. Various commercially available complex and synthetic media were assessed for their ability to promote germ tube forma-formation of *C. albicans* at 37°C. Washed yeast cells were inoculated into each medium at a concentration of 1.3 x 10^5 cfu/ml, incubated for 4 h, and evaluated for germ tube inducibility. As summarized in Table 2, only three media supported 90-100% germ tube formation: DMEM and HI-WO tissue culture media, and horse serum. Seven media induced up to 90% germ tube formation, while four media showed no germ tube formation. The four media in which germ tube formation did not occur did appear to support yeast cell growth since the percentage of budding cells increased and the turbidity of the culture fluid increased. Since one of the goals of this study was to determine the nutritional factors which promote germ tube formation, it was advantageous that DMEM and HI-WO tissue culture media were synthetic, chemically defined media. Because of the difficulty in obtaining HI-WO medium, we decided to examine more closely the ability of DMEM to

Table 2. Germ tube formation in various complex or synthetic media.

Medium[a]	Germ tube formation[b]
Sabouraud's Dextrose Broth	-
Yeast Nitrogen Base Broth (0.5-5.0%)	-
Casein Broth (0.5-5.0%)	-
Phytone Broth (0.5-5.0%)	-
Neopeptone	1+
Bactopeptone	1+
Malt Extract Broth	1+
Trypticase Soy Broth (BBL)	1+
Thioglycollate Medium	1+
Vitamin-free Casitone (5.0%)	1+
Vitamin-free Casitone (2.5%)	2+
Vitamin-free Casamino Acids	2+
Vitamin-free Casitone (1.0%)	2+
Vitamin-free Casitone (0.5%)	2+
Horse Serum (25-100%; Gibco)	3+
Dulbecco's Modified Eagle's Medium (Gibco)	3+
HI-WO (ISI)	3+

[a]All media, except those listed as obtained from Gibco or ISI, were obtained from Difco Laboratories (Detroit, MI) and prepared according to specified instructions unless otherwise noted.

[b]Yeast cells (1.3 x 10^5 cfu/ml) were incubated in the media for 4 h at 37°C. One hundred cells were counted. (-) = 0% germ tubes; 1+ = 1-50% germ tubes; 2+ = 50-90% germ tubes; 3+ = 90-100% germ tubes.

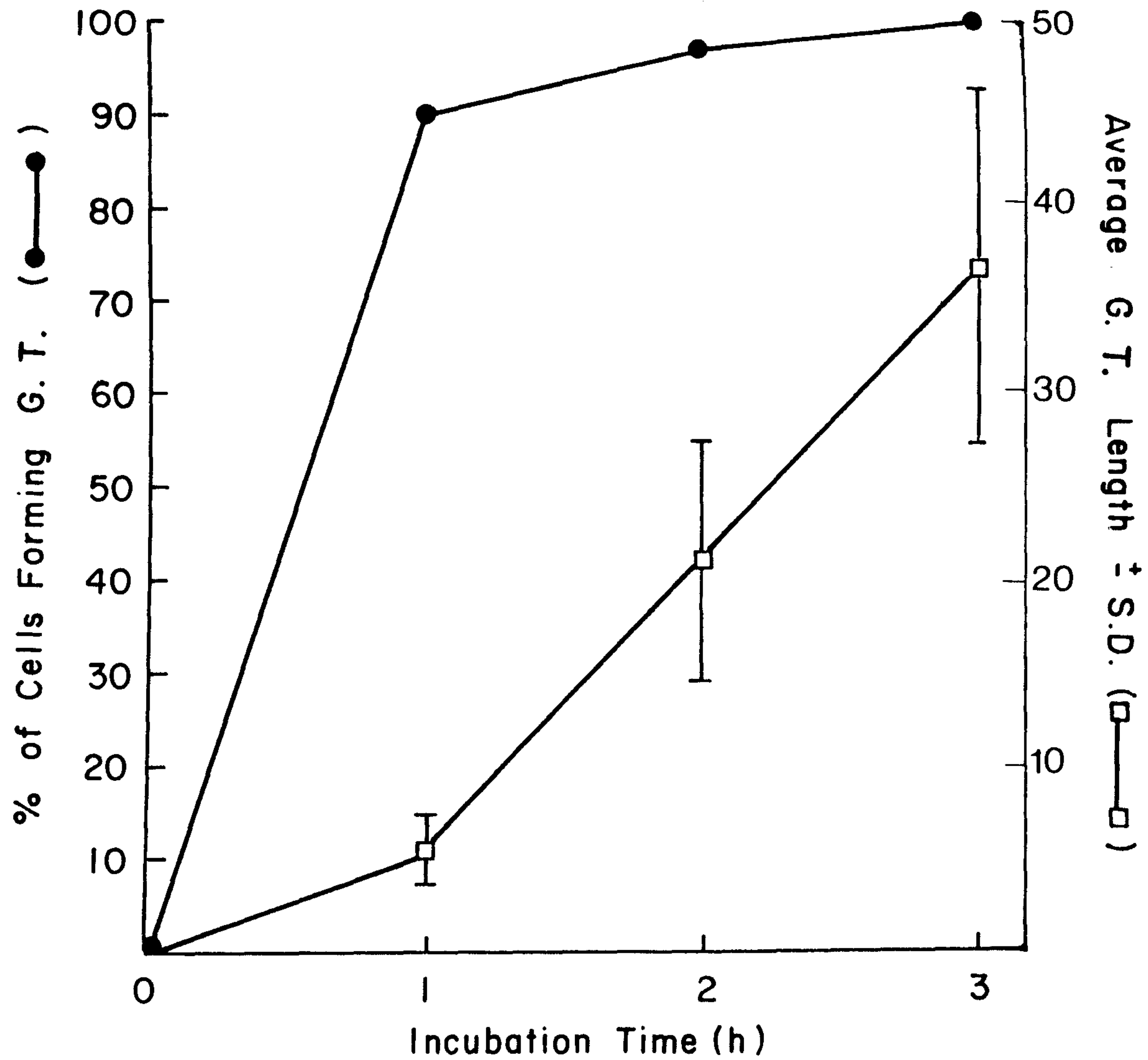
induce nearly complete germ tube formation.

Germ Tube Formation in DMEM.

1. Kinetics of germ tube formation in DMEM. *C. albicans* germ tube formation in DMEM was very rapid with 90% of the inoculum forming germ tubes after only 1 h incubation under standard conditions at 37°C (Fig. 2). After incubation for 3 h, complete germ tube formation had occurred. The length of the germ tubes appeared to increase linearly after 1 h incubation at a rate of approximately 15 µm/h. By 3 h incubation, hyphal length averaged 36.5 µm. After 8 h incubation in DMEM at 37°C, hyphae were approximately 100 µm in length and were multiseptated. But by this time, lateral bud formation began to occur at various points along the hyphae. When samples were scored for percent germ tube formation after 8 h incubation, the percent germ tubes decreased while the percent single and budded cells increased. This was probably due to the release of the single and budded cells from the hyphae during the vortexing performed prior to sampling. After 20 h incubation in DMEM at 37°C, the lengths of the hyphae were immeasurable due to their large size (greater than 200 µm) and their tendency to form hyphal clumps.

2. Germ tube formation in DMEM at various temperatures. An incubation temperature of 37°C was originally

Fig. 2. *C. albicans* germ tube formation in DMEM. Washed *C. albicans* yeast cells, 24 h old, were inoculated in DMEM (1.3×10^5 cfu/ml) and incubated at 37°C. Samples were removed hourly and examined microscopically for germ tube formation. Average germ tube length is expressed in micrometers. G.T. = germ tubes.

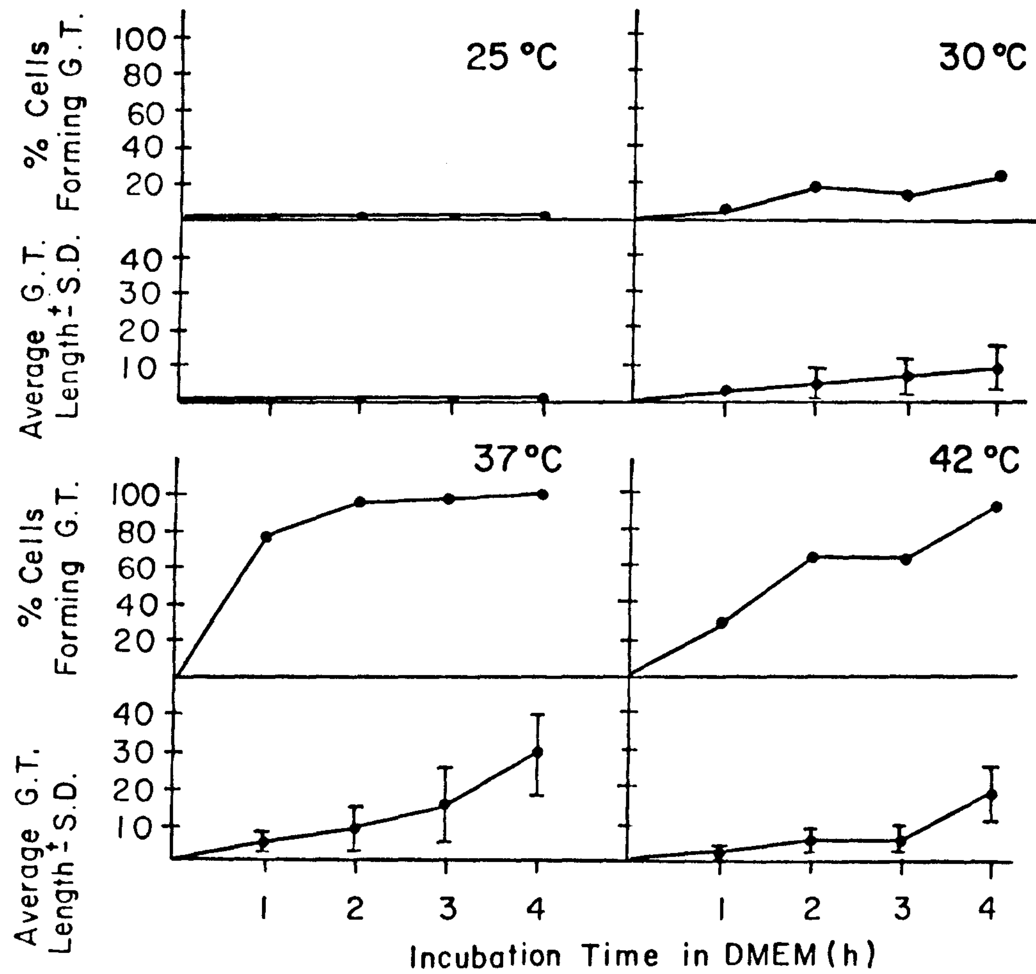


% of Cells Forming G. T.
Average G. T. Length ± S.D.
Incubation Time (h)
0
1
2
3
10
20
30
40
50
60
70
80
90
100

chosen because of the well-documented fact that serum could induce germ tube formation at that temperature (Taschjian et al., 1960). In order to determine if DMEM could induce germ tube formation at temperatures other than 37°C, we incubated yeast cells in DMEM under standard conditions at various temperatures. As demonstrated in Fig. 3, germ tube formation in DMEM appeared to be temperature dependent. No germ tube formation occurred at 25°C incubation though yeast cell replication appeared to take place since an increase in the percent budding and an increase in the turbidity of the culture fluid was observed. Only 20% germ tube formation occurred at 30°C incubation after 4 h. Nearly complete germ tube formation occurred at 37°C and at 42°C incubation. After 1, 2, and 3 h incubation, there was significantly more germ tube formation at 37°C than at 42°C ($p < 0.001$), as well as significantly greater germ tube lengths ($p < 0.01$). By 4 h incubation, germ tube formation at both temperatures was nearly complete, though the average hyphal length was still significantly greater at 37°C (30.5 µm vs. 18.3 µm, $p < 0.01$). Based on the results from this experiment, we chose 37°C as our standard incubation temperature.

3. Effect of pH on germ tube formation in DMEM. Germ tube formation in DMEM was studied over a wide pH range. When DMEM (normal pH = 7.2) was made acidic with 1.0 N HCl, nearly complete germ tube formation occurred down to a pH

Fig. 3. Germ tube formation of C. albicans in DMEM at various temperatures. C. albicans yeast cells were inoculated in DMEM under standard conditions, incubated at various temperatures, and examined for germ tube formation hourly for 4 h. Maximum germ tube formation and average germ tube length occurred at 37°C. Average germ tube length is expressed in micrometers. G.T. = germ tubes.


25 °C
30 °C
37 °C
42 °C
% Cells Forming G.T.
Average G.T. Length ± S.D.
100
80
60
40
20
40
30
20
10
1
2
3
4
Incubation Time in DMEM (h)

value of pH 3 (Table 3). When DMEM was made basic with 1.0 N NaOH, there was a decline in the percent germ tube formation even at pH 8. No germ tube formation was observed beyond pH 9. The inability of the cells to form germ tubes at values greater than pH 9 may have been due to the death of the cells in the NaOH-adjusted DMEM.

Using a series of different buffer systems, results similar to those found using the HCl- and NaOH-adjusted DMEM were obtained (Table 3). Nearly complete germ tube formation was observed between pH 5 and pH 8. A preliminary experiment showed that a twofold dilution of DMEM with distilled water had no effect on germ tube formation. Therefore, in the experimental procedure, DMEM was diluted twofold with each buffer tested. Although there seemed to be conflicting results between germ tube formation in acetate buffer and in Sorensen's citrate buffer at pH 4, this might be explained by the fact that pH 4 was the lowest limit for the buffering capacity of acetate buffer. At pH 4, the acetate buffer may have lost some of its buffering capacity and quite possibly prevented normal germ tube formation. Likewise, conflicting data obtained at pH 3 could be explained by the fact that the lowest limit for the buffering capacity of Sorensen's citrate buffer was pH 3. Since the pH values of the different buffer systems overlapped, the possibility that the increases or decreases in germ tube formation might have been due to an ion-related

Table 3. Effect of pH on germ tube formation in DMEM.[a]

Buffer	Percent cells forming germ tubes									
	pH									
	1	2	3	4	5	6	7	8	9	10
DMEM[b]	8	10	93	96	95	99	98	81	70	0
Sorensen's[c] citrate	-	-	47	93	-	-	-	-	-	-
Sodium[c] acetate	-	-	-	20	100	-	-	-	-	-
McIlvaine's[c] citrate	-	-	-	-	97	100	100	-	-	-
Sorensen's[c] phosphate	-	-	-	-	-	100	100	-	-	-
TMN[c]	-	-	-	-	-	-	100	100	-	-
Tris-HCl[c]	-	-	-	-	-	-	-	92	-	-

[a]Stationary phase C. albicans yeast cells were inoculated into DMEM under standard conditions. Percent germ tube formation was determined after 4 h incubation at 37°C.

[b]The pH of DMEM was adjusted using 1.0 N HCl or 1.0 N NaOH.

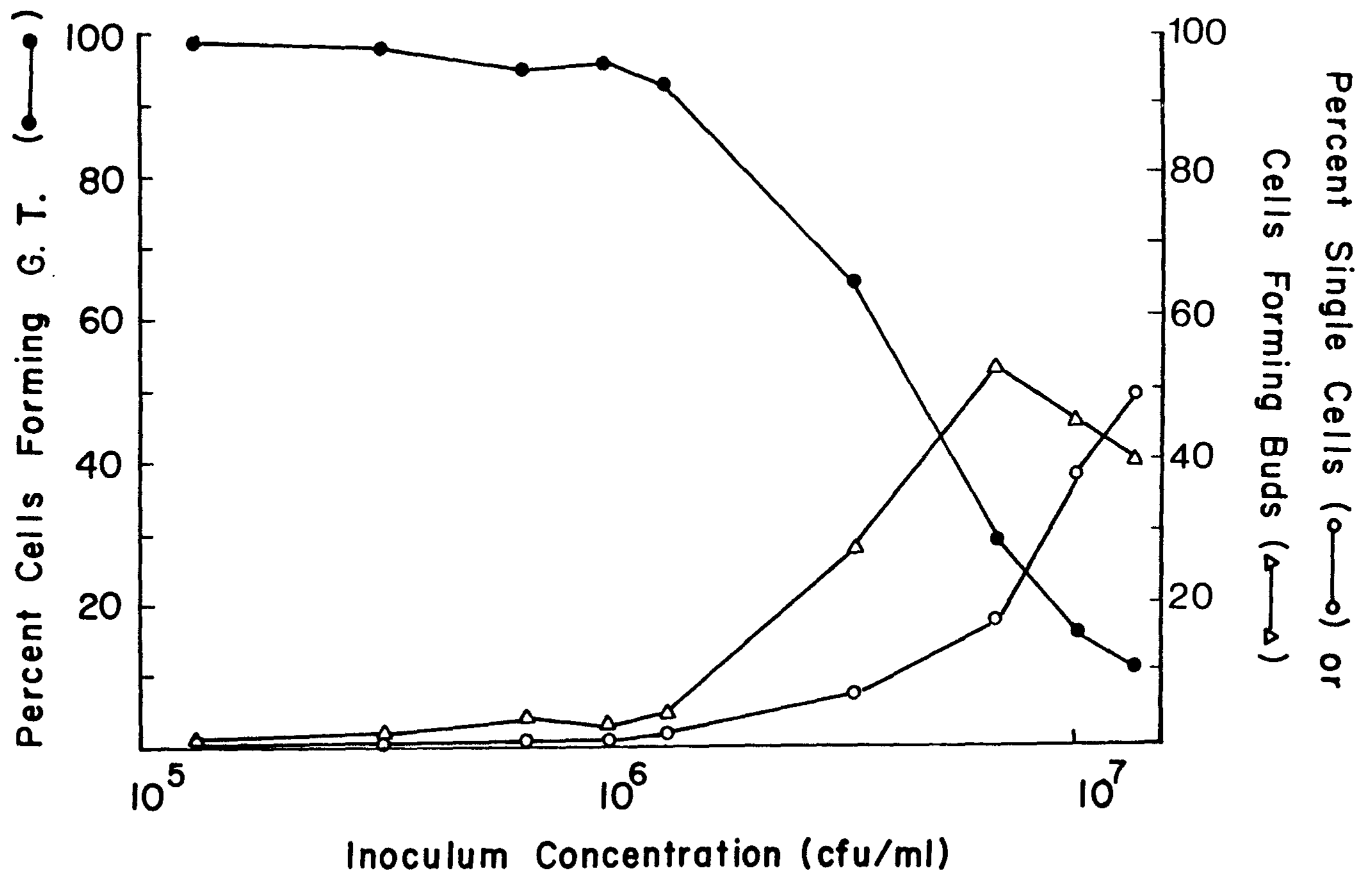
[c]The pH of DMEM was adjusted by diluting DMEM 1:1 with the appropriate buffer (final buffer molarity = 0.05 M).

effect was ruled out. Since DMEM buffered with TMN pH 7 yielded 100% germ tube formation with the maximum average hyphal length (55.9 µm) after 4 h incubation at 37°C, TMN buffer pH 7 was used as the diluent in later experiments.

4. Effect of inoculum concentration on germ tube formation in DMEM. Germ tube formation in DMEM also appeared to be dependent on the inoculum concentration (Fig. 4). Nearly complete germ tube formation occurred at inoculum concentrations between 1.3×10^5 cfu/ml and 1.3×10^6 cfu/ml. At inoculum concentrations higher than 1.3×10^6 cfu/ml, there was a steady decrease in the amount of germ tube formation to 11% by 1.3×10^7 cfu/ml. As the percentage of germ tube forming cells decreased at higher inoculum concentrations, there was an increase in the percentage of budding and single cells. Since the initial percentage of budding cells was less than 15%, this increase in the proportion of budding cells at the higher inoculum concentrations suggested that active yeast cell replication was occurring. From the results of this experiment, 1.3×10^5 cfu/ml was selected as the standard inoculum concentration.

5. Elucidation of DMEM germ tube inducing factor(s). We wanted to determine exactly the component(s) of DMEM responsible for germ tube formation. As mentioned previously, it was advantageous that DMEM induced approxi-

Fig. 4. Germ tube induction of *C. albicans* in DMEM at various inoculum concentrations. Yeast cells (1.3 x 10^5 cfu/ml) were incubated in DMEM at 37°C. Cell counts were determined after 4 h incubation. Note the significant decrease in germ tube induction at inoculum sizes greater than 10^6 cfu/ml. G.T. = germ tubes.



Percent Cells Forming G. T. (●—●)
100
80
60
40
20
Percent Single Cells (○—○) or
Cells Forming Buds (△—△)
100
80
60
40
20
10^5
10^6
10^7
Inoculum Concentration (cfu/ml)

mately 100% germ tube formation. Since DMEM was a synthetic medium, it was possible to prepare it in the laboratory. As seen in Table 4, DMEM can be divided into four major groups: salts, glucose, amino acids, and vitamins. By preparations of stock solutions of these groups, components necessary for germ tube formation could be determined through elimination and reconstitution studies. Since commercially available BME Amino Acid Solution contained all the amino acids found in DMEM except glycine and serine, this solution was used as the amino acid stock solution. Amino acid concentrations (1x) in the BME Amino Acid Solution were approximately one-fourth of those found in DMEM. Similarly, BME Vitamin Solution contained all the vitamins found in DMEM, with biotin in addition. Vitamin concentrations (1x) in the BME Vitamin Solution were all 2.5 times higher than in DMEM. Stock solutions of the salts (10x) in distilled water were prepared according to the composition of DMEM (Table 4). A $CaCl_2$ stock solution (10x) was prepared separately to prevent precipitation of the phosphate. Glucose stock solution (10x) was prepared at a concentration of 55.5 mM (1.0%). TMN buffer (0.05 M, pH 7.0) was used as the diluent. The only two components of DMEM which our stock solutions lacked were sodium pyruvate and phenol red.

Reconstitution studies using the aforementioned stock solutions suggested that amino acids were mainly responsi-

Table 4. Composition of DMEM, BME amino acid solution, and BME vitamin solution.[a]

Component	DMEM mg/1 (mM)	BME amino acid solution mg/1 (mM)	BME vitamin solution mg/1 (mM)
$CaCl_2$	200.00 (1.802)	-	-
$Fe(NO)_3 \cdot 9H_2O$	0.10 (0.003)	-	-
KCl	400.00 (5.366)	-	-
$MgSO_4 \cdot 7H_2O$	200.00 (0.812)	-	-
NaCl	6400.00 (109.5)	-	-
$NaHCO_3$	3700.00 (44.05)	-	-
$NaHPO_4 \cdot 7H_2O$	125.00 (0.492)	-	-
Glucose	1000.00 (5.551)	-	-
Phenol red	15.00 (0.042)	-	-
Sodium pyruvate	110.00 (1.170)	-	-
Arginine HCl	84.00 (0.398)	21.00 (0.100)	-
Cystine	48.00 (0.200)	12.00 (0.050)	-
Glutamine	584.00 (4.000)	292.00 (2.000)	-

Table 4. continued

Component	DMEM mg/l (mM)	BME amino acid solution mg/l (mM)	BME vitamin solution mg/l (mM)
Glycine	30.00 (0.400)	-	-
Histidine	-	8.00 (0.052)	-
Histidine HCl·H_2O	42.00 (0.219)	-	-
Isoleucine	105.00 (0.766)	26.00 (0.190)	-
Leucine	105.00 (0.766)	26.00 (0.190)	-
Lysine HCl	146.00 (0.799)	36.24 (0.200)	-
Methionine	30.00 (0.201)	7.50 (0.050)	-
Phenylalanine	66.00 (0.400)	16.50 (0.100)	-
Serine	42.00 (0.400)	-	-
Threonine	95.00 (0.798)	24.00 (0.202)	-
Tryptophane	6.00 (0.078)	4.00 (0.020)	-
Tyrosine	72.00 (0.397)	18.00 (0.099)	-
Valine	94.00 (0.802)	23.50 (0.201)	-
Biotin	-	-	10.00 (0.041)

Table 4. continued

Component	DMEM mg/l (mM)	BME amino acid solution mg/l (mM)	BME vitamin solution mg/l (mM)
D-Ca pantothenate	4.00 (0.008)	-	10.00 (0.021)
Choline chloride	4.00 (0.029)	-	10.00 (0.072)
Folic acid	4.00 (0.009)	-	10.00 (0.023)
i-inositol	7.20 (0.040)	-	18.00 (0.100)
Nicotinamide	4.00 (0.033)	-	10.00 (0.082)
Pyridoxal HCl	4.00 (0.020)	-	10.00 (0.049)
Riboflavin	0.40 (0.001)	-	1.00 (0.003)
Thiamine HCl	4.00 (0.012)	-	10.00 (0.029)

[a]Data from the Gibco catalogue (3/82).

ble for germ tube formation in DMEM (Table 5). One hundred percent germ tube formation occurred when yeast cells were incubated at 37°C in the reconstituted DMEM (BME amino acids + salts + BME vitamins + glucose) as well as in DMEM itself. BME amino acids alone promoted 58% germ tube formation. The addition of salts or glucose to the amino acid stock increased the percentages to 88% and 84%, respectively. After this work was completed, it was shown that the small amount of germ tube formation in glucose or salts alone, was, in fact, due to a previously unknown contamination of the commercial buffer with ethanol (Table 13; Pollack and Hashimoto, 1984). The addition of vitamins to amino acids or to amino acids plus salts did not increase the amount of germ tube formation. It was apparent that addition of salts was necessary to promote optimum germ tube formation, and therefore, further experiments included the addition of salts to the amino acids.

Since the amino acid composition of the BME Amino Acid Solution was known, single amino acid stock solutions were prepared at concentrations found in DMEM and used in similar reconstitution studies. When washed C. albicans yeast cells were inoculated into each single amino acid medium and incubated at 37°C, it was observed (Table 6) that, even after eight hours incubation, of the 12 amino acids found in the BME Amino Acid Solution, only arg and gln supported substantial germ tube formation (80% and 75%, re-

Table 5. Germ tube formation of *C. albicans* in the various components of DMEM.[a]

Composition of medium	% cells forming germ tubes
DMEM	100
BME amino acids + salts + BME vitamins + glucose	100
BME amino acids	58
BME amino acids + salts	88
BME amino acids + BME vitamins	62
BME amino acids + glucose	83
BME amino acids + salts + BME vitamins	90
BME amino acids + salts + glucose	95
BME amino acids + BME vitamins + glucose	49
Salts + BME vitamins + glucose	21
Glucose	17
Salts	16
BME vitamins	0

[a] Stationary phase *C. albicans* yeast cells were inoculated into each sample (1.3×10^5 cfu/ml) and incubated at 37°C for 4 h. For exact protocol and final concentraions, see Materials and Methods.

Table 6. Germ tube formation of *C. albicans* in single amino acid media.[a]

Amino acid (mM)	Percent cells forming germ tubes			
	Incubation time (h)			
	2	4	6	8
Arg (0.398)	44	69	78	80
Cys (0.200)	4	13	19	22
Gln (4.000)	30	62	66	75
His (0.219)	3	15	18	34
Ile (0.766)	6	7	9	15
Leu (0.766)	7	9	16	14
Lys (0.799)	9	15	21	20
Met (0.201)	9	7	15	8
Phe (0.400)	6	14	15	15
Thr (0.798)	7	18	24	26
Tyr (0.397)	5	10	15	18
Val (0.400)	1	15	21	20
Salts	1	6	6	18
DMEM	93	99	99	100

[a] Each medium consisted of the amino acids and salts (at concentrations found in DMEM) in TMN buffer. Cells were inoculated and incubated under standard culture conditions. Except for arg, gln, and DMEM samples, all germ tubes were less than 10 µm in length.

spectively). All other amino acids did not show any more germ tube formation than that found in the salts control. Maximum germ tube formation in arg and gln seemed to occur by 4 h incubation at 37°C and extended incubation did not substantially increase the percentages. As will be seen in later experiments, arg and gln media never induced greater than 85% germ tube formation under standard conditions. Glycine and serine, the two amino acids found in DMEM but not in the BME Amino Acid Solution, as well as D-arg and D-gln, did not induce any germ tube formation under similar experimental conditions (data not shown). Also, incubation of yeast cells in DMEM without phenol red resulted in nearly complete germ tube formation suggesting that the indicator did not play a role in germ tube formation (data not shown).

Germ Tube Formation in Arg and Gln Media.

1. Kinetics of germ tube formation in arg and gln media. Arg and gln media were further tested for their germ tube inducing capabilities. In a representative experiment, arg and gln media induced 55% and 60% germ tube formation, respectively, after 4 h incubation at 37°C (Table 7). The percent germ tube formation in these two single amino acid media tested under standard conditions varied on a daily basis, but always fell in the range of 50% to 85%. As noted in a previous experiment (Table 6),

Table 7. Kinetics of germ tube formation in arg and gln media.[a]

Incubation time (h)	Arg		Gln	
	% cells forming germ tubes	Average germ tube length(μm)	% cells forming germ tubes	Average germ tube length(μm)
0	0	-	0	-
1	13	2.7 ± 0.7	20	2.9 ± 0.9
2	30	4.4 ± 1.9	39	4.0 ± 2.0
3	48	6.5 ± 4.2	54	5.7 ± 4.4
4	55	10.5 ± 6.8	60	9.6 ± 6.7

[a] Stationary phase yeast cells were inoculated into arg or gln media under standard conditions and incubated at 37°C. Samples were removed hourly and percent germ tubes and average germ tube length were determined.

extended incubation times did not increase the percentages. The average germ tube lengths in arg and gln media after 4 h incubation were 10.0 µm and 9.6 µm, respectively. Under standard conditions, the average germ tube length in either media also varied on a daily basis, but never exceeded 25 um. As in DMEM, by 8 h incubation, lateral bud formation occurred suggesting that the media could still support yeast cell replication and growth, but not germ tube formation. Interestingly, germ tube forming cells in gln occasionally demonstrated up to three germ tubes extending from the parent yeast cell.

It is important to mention that nearly complete germ tube formation occurred by 3 h incubation in the DMEM control indicating that every cell in the inoculum had the capability to form germ tubes.

2. Effect of arg and gln concentration on germ tube formation. The DMEM concentrations of arg and gln were 0.4 mM and 4.0 mM, respectively. Increasing the amino acid concentration in arg or gln media to 40 mM did not increase the amount of germ tube formation in either of the two media (Table 8). Also, a decrease in the percent germ tube formation was observed only when the amino acid concentration in either medium was decreased 1000-fold or greater from that of the normal DMEM concentration.

3. Effect of inoculum concentration on germ tube formation in arg and gln media. Washed yeast cells were ino-

Table 8. Effect of arg or gln concentration on germ tube induction in *C. albicans*.[a]

Inducer concentration (mM)	Percent cells forming germ tubes	
	Arg	Gln
40.0	80	80
4.0	81	75
0.4	81	81
0.04	86	70
0.004	74	57
0.0004	20	4
0.00004	0	0

[a] Stationary phase *C. albicans* yeast cells were inoculated into the various media and incubated under standard conditions. Percent germ tube formation was determined after 4 h incubation at 37°C.

culated into arg and gln media at various concentrations and incubated at 37°C. As shown in Fig. 5, germ tube formation in arg medium was dependent on the inoculum concentration. In our system, maximum germ tube formation occurred at an inoculum concentration of 1.3×10^5 cfu/ml. Inoculum concentrations lower than this were not studied due to the fact that increased dilution of the cells resulted in an inability to microscopically count a significant number of cells. Any increase in the inoculum concentration above 1.3×10^5 cfu/ml correspondingly decreased the percent germ tube formation to the point that, by 1.3×10^7 cfu/ml, no germ tube formation took place. Also, the proportion of budding cells remained relatively constant (10-21%) over the range of inoculum concentrations while the proportion of single cells increased (7-79%). This suggested that active yeast cell replication was not occurring in samples with higher inoculum concentrations. Similar results were seen in gln medium (Fig. 6).

4. Effect of pH on germ tube formation in arg and gln media. The pH of the arg and gln media was varied by preparing the single amino acid media using various buffers. A pH range of pH 3 to pH 10 was covered by the buffers. When compared to the control media prepared with TMN buffer pH 7, normal percentages of germ tube formation in arg and gln media occurred over a pH range of pH 5 to pH 9 (Tables 9 and 10). Decreased percentages of germ tube formation

Fig. 5. Germ tube induction of *C. albicans* in arg (0.398 mM) medium over various inoculum concentrations. Cells were inoculated under standard conditions and germ tubes were counted after 4 h incubation at 37°C. Inoculum sizes lower than 10^5 cfu/ml were not studied for technical reasons. Note the increase in the proportion of single yeast cells as the inoculum size increased, while the number of budding yeast cells remained relatively constant. G.T. = germ tubes.

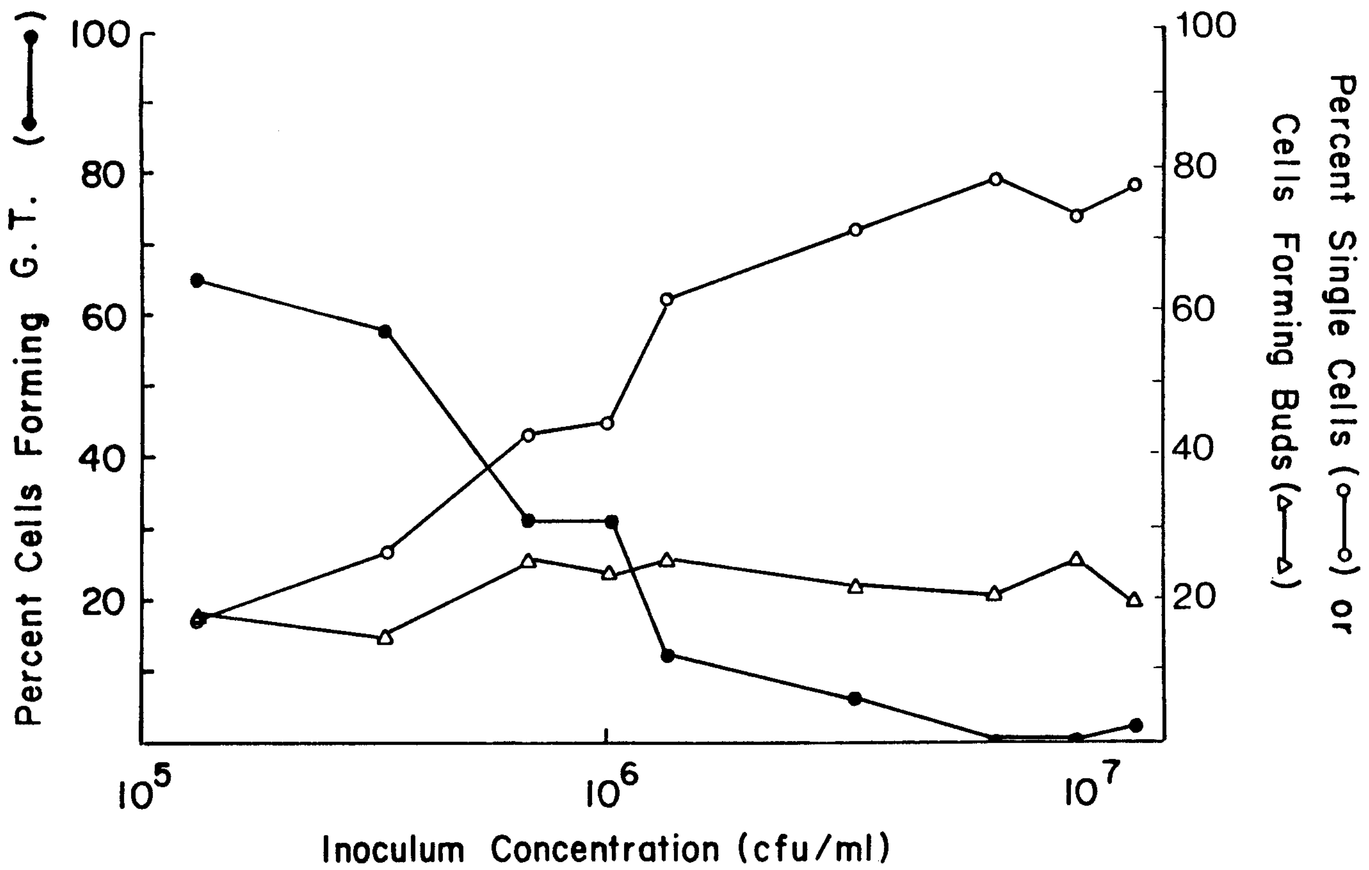

Percent Cells Forming G. T. (●—●)
100
80
60
40
20
Percent Single Cells (○—○) or
Cells Forming Buds (△—△)
100
80
60
40
20
10^5
10^6
10^7
Inoculum Concentration (cfu/ml)

Fig. 6. Germ tube induction of C. albicans in gln (4.00 mM) medium over various inoculum concentrations. Cells were inoculated under standard conditions and germ tubes were counted after 4 h incubation at 37°C. Inoculum sizes lower than 10^5 cfu/ml were not studied for technical reasons. Note the increase in the proportion of single yeast cells as the inoculum size increased, while the number of budding yeast cells remained relatively constant. G.T. = germ tubes.

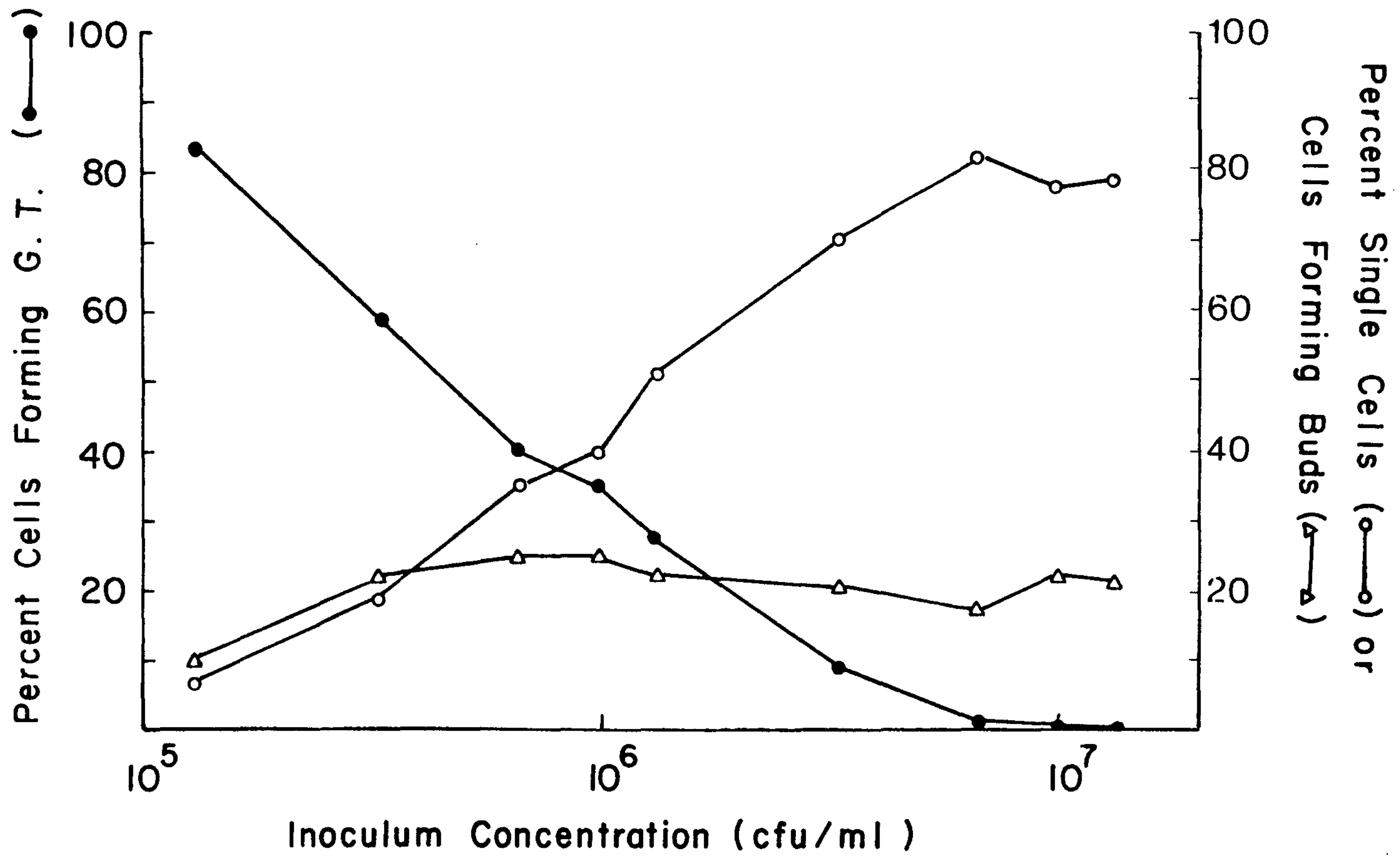

Percent Cells Forming G. T. (●—●)
100
80
60
40
20
Percent Single Cells (○—○) or
Cells Forming Buds (△—△)
100
80
60
40
20
10^5
10^6
10^7
Inoculum Concentration (cfu/ml)

Table 9. Effect of pH on germ tube formation in arg medium.[a]

Buffer[b]	Percent cells forming germ tubes								
	pH								
	3	3.5	4	5	6	7	8	9	10
Sorensen's citrate	0	12	27	-	-	-	-	-	-
Sodium acetate	-	-	16	58	-	-	-	-	-
McIlvaines's citrate	-	-	-	58	61	70	-	-	-
Sorensen's phosphate	-	-	-	-	59	62	-	-	-
TMN	-	-	-	-	-	59	55	-	-
Tris-HCl	-	-	-	-	-	-	60	-	-
Smith and Smith's piperazine	-	-	-	-	-	-	-	55	11

[a]Stationary phase C. albicans yeast cells were inoculated into arg medium under standard conditions. Percent germ tubes was determined after 4 h incubation at 37°C.

[b]The pH of the arg medium was adjusted by diluting 1:1 with buffer (final buffer molarity = 0.05 M).

Table 10. Effect of pH on germ tube formation in gln medium.[a]

Buffer[b]	Percent cells forming germ tubes								
	pH								
	3	3.5	4	5	6	7	8	9	10
Sorensen's citrate	0	0	30	-	-	-	-	-	-
Sodium acetate	-	-	0	47	-	-	-	-	-
McIlvaine's citrate	-	-	-	64	70	67	-	-	-
Sorensen's phosphate	-	-	-	-	71	69	-	-	-
TMN	-	-	-	-	-	71	70	-	-
Tris-HCl	-	-	-	-	-	-	72	-	-
Smith's and Smith's piperazine	-	-	-	-	-	-	-	65	21

[a]Stationary phase _C. albicans_ yeast cells were inoculated into gln medium under standard conditions. Percent germ tubes was determined after 4 h incubation at 37°C.

[b]The pH of the gln medium was adjusted by diluting 1:1 with buffer (final buffer molarity = 0.05 M).

were seen at pH values lower than pH 5 or higher that pH 9.

5. Supplementation of arg and gln media. Since BME Amino Acid Solution in the presence of salts promoted nearly complete germ tube formation, we decided to supplement arg and gln media with the various amino acids also found in the commercial amino acid solution and monitor germ tube formation. Percent germ tube formation in the arg and gln control tubes was 61% and 66%, respectively (Table 11). The combination of the two amino acids only slightly raised the percentage to 74%. When all twelve amino acids found in BME Amino Acid Solution were prepared separately at concentrations found in DMEM and added in combination, 72% germ tube formation occurred. Deletion of arg or gln or both from the reconstituted amino acid solution resulted in a slight decrease in germ tube formation. Interestingly, addition of valine (val) to the single amino acid media significantly decreased the percent germ tube formation. Addition of val to two other inducers of our *C. albicans* strain, proline (0.01 mM) and N-acetyl glucosamine (2.5 mM), did not inhibit germ tube formation. Addition of any of the other ten amino acids found in BME Amino Acid Solution to either arg or gln medium did not significantly increase or decrease the amount of germ tube formation (data not shown).

As seen in the original reconstitution study (Table 5), glucose supplementation of BME Amino Acid Solution ap-

Table 11. Germ tube formation by various combinations of amino acids.

Amino acids added[a]	% cells forming germ tubes
Arg	61
Gln	66
Arg + Gln	74
complete	72
complete - Arg	66
complete - Gln	67
complete - Arg, Gln	52
Val	0
Arg + Val	41
Gln + Val	38
complete - Val	79
Pro (0.01 mM)[b]	99
Pro + Val	94
GlcNAc (2.5 mM)[b]	98
GlcNAc + Val	86

[a] Yeast cells were inoculated into tubes containing the various amino acids in TMN buffer and incubated for 4 h at 37°C. Results are the average of duplicate samples. Amino acid concentrations were similar to those found in DMEM. The complete medium consisted of all thirteen amino acids found in BME amino acid solution.

[b] Pro = Proline; GlcNAc = N-acetylglucosamine.

peared to increase the amount of germ tube formation. Supplementation of arg and gln media with 0.1% glucose did not not significantly increase the percent germ tube formation (Table 12). Yet, in the TMN buffer control, there seemed to be some measurable germ tube formation. Since, throughout the study, we consistently obtained between 0% and 10% germ tube formation in the TMN buffer control, we decided to examine this phenomenon more closely. TMN buffer was prepared using two different methods. In the first method (used consistently throughout this study), the buffer was prepared using Tris maleate (Sigma). In the second, a combination of Tris base (Sigma) and maleic acid (Mallinkcrodt) was used. As can be seen in Table 13, spontaneous germ tube formation occurred in TMN buffer prepared with Tris maleate, especially in the presence of glucose and salts. The amount of spontaneous germ tube formation which occurred in TMN buffer prepared by the second method was significantly less that that by the first method. Also, the amount of spontaneous germ tube formation in 0.067 M phosphate buffer (pH 7) was equal to that found in Tris/maleic acid TMN buffer. This data suggested that the original TMN buffer prepared using the Tris maleate was contaminated with a substance which induced germ tube formation. Work done by another group in this laboratory has since shown that, indeed, the commercially prepared Tris maleate was contaminated with ethanol, which, in the pre-

Table 12. Effect of glucose supplementation on arg or gln induced germ tube formation in *C. albicans*.[a]

Medium	Percent cells forming germ tubes	
	Without addition of 0.1% glucose	With addition of 0.1% glucose
DMEM	99	N.D.[b]
TMN buffer	3	16
Arg	56	61
Gln	66	69

[a] Washed stationary phase yeast cells (1.3 x 10^5 cfu/ml) were inoculated under standard conditions into each medium. Each medium was prepared with and without the addition of 0.1% glucose. Samples were removed and examined microscopically after 4 h incubation at 37°C.

[b] N.D. = Not determined.

Table 13. Spontaneous germ tube induction of *C. albicans* in buffer and glucose.[a]

Buffer	Glucose (%)	Percent cells forming germ tubes	
		without salts	with salts
TMN[b] (0.02 M, pH 7)	0	0	2
	0.1	3	13
	0.5	6	5
	1.0	0	20
	2.0	2	40
	3.0	0	20
	4.0	0	24
TMN[c] (0.02 M, pH 7)	0	0	0
	0.1	0	6
	0.5	0	2
	1.0	0	3
	2.0	0	8
	3.0	0	6
	4.0	0	1
Phosphate (0.067 M, pH 7)	0	0	0
	0.1	0	0
	0.5	0	3
	1.0	0	4
	2.0	0	4
	3.0	0	0
	4.0	0	0

[a] Stationary phase *C. albicans* yeast cells were inoculated under standard conditions and incubated in glucose (Mallinkcrodt) with or without salts for 4 h at 37°C. Results of the same experiment using glucose from other sources were similar.

[b] Prepared using Trizma (Tris) maleate (Sigma).

[c] Prepared using Trizma (Tris) base (Sigma) and maleic acid (Mallinkcrodt).

sence of salts, could induce a measurable amount of germ tube formation (Pollack and Hashimoto, 1984).

6. Effect of inoculum preincubation on germ tube formation in arg and gln media. Since we were unsuccessful in raising the percent germ tube formation in arg and gln media by supplementation, we felt that, by some type of inoculum manipulation, we might increase the amount of germ tube formation to 100%. Washed *C. albicans* yeast cells (1.3×10^6 cfu/ml) were preincubated in TMN buffer for 12 h at various temperatures and then assessed for their ability to form germ tubes in arg and gln media. The results in Tables 14 and 15 demonstrate that preincubation of the inoculum at 15°C, 20°C, and 25°C enhanced the ability of the cells to form germ tubes in the single amino acid media. Indeed, nearly complete germ tube formation in the single amino acid media was obtained after this preincubation. Microscopic examination of the preincubated inoculum showed no change in the morphological profile of the cells. When the inoculum cells were incubated in TMN buffer at 20°C and assessed at various intervals for germ tube formation, the cells appeared to acquire this enhancement by 6 h preincubation (Tables 16 and 17). Most striking was the amount of germ tube formation observed even after only 1 h incubation in arg or gln media. Though a greater proportion of cells formed germ tubes, the average germ tube length did not increase when compared to that of

Table 14. Enhancement of arg induced germ tube formation by preincubation of inoculum in TMN buffer at various temperatures.[a]

Preincubation temperature (°C)	% cells forming germ tubes ± S.D.[b]		
	Incubation time in arg		
	2	4	6
Control (no preincubation)	48.4 ± 10.3	65.8 ± 17.3	73.0 ± 19.1
37	47.3 ± 22.5	75.0 ± 20.3	84.8 ± 11.5
25	74.0 ± 12.5*	89.5 ± 5.8*	95.9 + 3.1*
20	89.8 ± 14.7*	90.5 ± 6.2*	90.5 ± 6.2
15	88.0 ± 4.2*	93.0 ± 1.8*	96.0 ± 0.8*
6	66.8 ± 13.9*	82.3 ± 9.0	81.8 ± 9.0

[a] Washed stationary phase _C. albicans_ yeast cells were suspended in TMN buffer (pH 7) at a concentration of 1.3 x 10^6 cfu/ml and preincubated for 12 h at various temperatures. After the preincubation period, cells were directly inoculated into arg medium (1.3 x 10^5 cfu/ml) and incubated at 37°C.

[b] Values represent the data from four separate experiments. Asterisks indicate those values statistically significant as compared to the control (Student's t-test, $p<0.025$).

Table 15. Enhancement of gln induced germ tube formation by preincubation of inoculum in TMN buffer at various temperatures.[a]

Preincubation temperature (°C)	% cells forming germ tubes ± S.D.[b] Incubation time in gln 2	4	6
Control (no preincubation)	33.4 ± 7.8	55.8 ± 13.2	64.4 ± 14.9
37	53.0 ± 25.6	71.5 ± 12.1	74.3 ± 12.6
25	75.0 ± 15.5*	89.0 ± 10.2	93.5 ± 5.9*
20	82.0 ± 6.9*	85.5 ± 3.7*	88.3 ± 5.0*
15	83.3 ± 6.4*	89.3 ± 7.5*	88.0 ± 10.6*
6	67.3 ± 16.6*	75.8 ± 7.9*	80.5 ± 11.0

[a] Washed stationary phase *C. albicans* yeast cells were suspended in TMN buffer (pH 7) at a concentration of 1.3 x 10^6 cfu/ml and preincubated for 12 h at various temperatures. After the preincubation period, cells were directly inoculated into gln medium (1.3 x 10^5 cfu/ml) and incubated at 37°C.

[b] Values represent the data from four separate experiments. Asterisks indicate those values statistically significant as compared to the control (Student's t-test, $p<0.025$).

Table 16. Enhancement of arg induced germ tube formation by preincubation of inoculum in TMN buffer at 20°C.[a]

Preincubation time in TMN buffer (h)	% cells forming germ tubes ± S.D.[b]		
	Incubation time in arg (h)		
	1	2	4
0	15.5 ± 15.9	64.3 ± 14.0	83.3 ± 5.4
1.5	25.0 ± 29.5	71.3 ± 14.6	83.0 ± 10.3
3	42.3 ± 35.2	76.0 ± 12.9	84.3 ± 14.2
6	58.8 ± 7.7*	85.3 ± 16.3	90.5 ± 11.2
9	66.3 ± 34.9*	87.8 ± 16.5	90.0 ± 14.7
12	55.5 ± 26.8*	84.8 ± 14.2	88.0 ± 14.0

[a] Washed stationary phase *C. albicans* yeast cells were suspended in TMN buffer (pH 7) at a concentration of 1.3 x 10^6 cfu/ml and incubated at 20°C for various lengths of time. After the preincubation period, cells were directly inoculated into arg medium (1.3 x 10^5 cfu/ml) and incubated at 37°C.

[b] Values represent the data from four separate experiments. Asterisks indicate those values statistically significant as compared to the 0 h control (Student's t-test, $p<0.05$).

Table 17. Enhancement of gln induced germ tube formation by preincubation of inoculum in TMN buffer at 20°C.[a]

Preincubation time in TMN buffer (h)	% cells forming germ tubes ± S.D.[b]		
	Incubation time in gln (h)		
	1	2	4
0	24.0 ± 11.3	60.5 ± 13.5	83.3 ± 5.4
1.5	26.3 ± 25.5	72.5 ± 12.7	85.0 ± 7.1
3	44.8 ± 29.9	72.5 ± 13.9	85.3 ± 12.1
6	60.3 ± 23.0*	86.6 ± 16.4	87.0 ± 15.9
9	68.8 ± 18.1*	86.3 ± 12.8	90.8 ± 10.6
12	57.0 ± 25.2*	81.5 ± 18.9	83.8 ± 18.8

[a] Washed stationary phase *C. albicans* yeast cells were suspended in TMN buffer (pH 7) at a concentration of 1.3 x 10^6 cfu/ml and incubated at 20°C for various lengths of time. After the preincubation period, cells were directly inoculated into gln medium (1.3 x 10^5 cfu/ml) and incubated at 37°C.

[b] Values represent the data from four separate experiments. Asterisks indicate those values statistically significant as compared to the 0 h control (Student's t-test, $p<0.05$).

non-preincubated cells (data not shown). It would appear that there were conflicting results between Tables 14 and 15 and Tables 16 and 17 since the latter did not show a significant increase in germ tube formation by 4 h incubation in the single amino acid media using the preincubated inoculum. This could be explained by the fact that there was a daily variation in the percent germ tube formation and the percent germ tube formation in the control in Tables 16 and 17 was high enough to show no significance.

In the previous experiments, preincubated inoculum cells in TMN buffer were directly inoculated into the single amino acid media. In a separate experiment, the preincubated inoculum cells were washed by Millipore filtration with fresh TMN buffer, resuspended in an equal volume of fresh TMN buffer, and then inoculated into arg and gln media. These cells also demonstrated an enhanced ability to form germ tubes in the single amino acid media (data not shown). This suggested that the increased germ tube formation after preincubation was not caused by the secretion of an inducing factor by the yeast cells during the preincubation period.

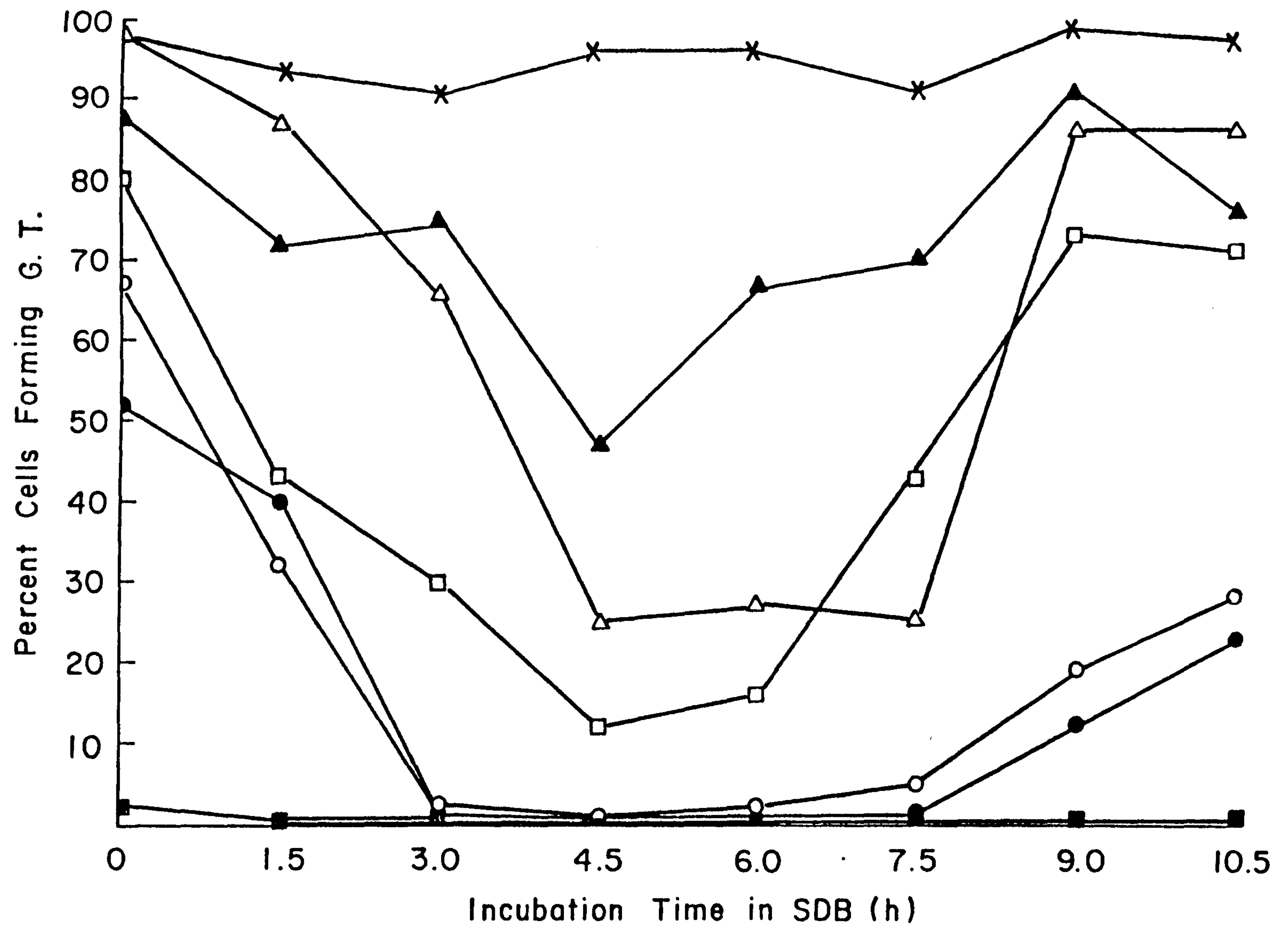
Effect of Growth Phase of the Inoculum on Germ Tube Formation. Work done by other groups has suggested that the growth phase of the inoculum is critical for germ tube formation. We examined the effect of the growth phase of the

inoculum on germ tube formation in our induction system. Yeast cells were incubated in SDB to various points in the growth curve and assessed for their ability to form germ tubes. Fig. 7 illustrates the results from a representative experiment. Cells incubated in SDB for a period of 1.5 h to 7.5 h were typically in lag phase whereas cells sampled between 7.5 h and 10.5 h were typically in early log phase. Cells in lag phase appeared to lose their ability to form germ tubes in arg and gln media. When lag phase cells were inoculated into BME Amino Acid Solution, proline or GlcNAc media, there was alo a significant decrease in the amount of germ tube formation. When early log phase cells were inoculated into the five media, the cells seemed to regain their germ tube forming capability. Germ tube formation in DMEM remained nearly complete regardless of the growth phase of the inoculum.

Prior to inoculation into the germ tube induction media, the morhological profile of the inoculum was determined. Nearly 100% of the cells incubated in SDB to lag phase or early log phase were budded cells. Since complete germ tube formation in DMEM was obtained using the budded cells, this suggested that germ tube formation could take place regardless of the point in the growth phase of the inoculum cells.

Cells which were grown to various points in the growth curve were also washed in TMN buffer, preincubated at

Fig. 7. Effect of growth phase of inoculum on germ tube induction of C. albicans. Cells were incubated in SDB for various lengths of time, washed in TMN buffer, and inoculated into the various media under standard conditions. Germ tubes were counted after 4 h incubation at 37°C in the presence of specified inducers. Germ tube induction of early log phase cells appeared to decrease in all the media tested, and returned to normal levels for mid-log phase cells. Symbols: (●—●) = arg; (○—○) = gln; (■—■) = TMN buffer; (□—□) = BME amino acid solution; (Δ—Δ) = pro; (▲—▲) = GlcNAc; (×—×) = DMEM; G.T. = germ tubes.


Percent Cells Forming G. T.
100
90
80
70
60
50
40
30
20
10
0
1.5
3.0
4.5
6.0
7.5
9.0
10.5
Incubation Time in SDB (h)

$20^{\circ}C$ for 12 h, and then inoculated into arg and gln media. Preincubation of early log phase cells at $20^{\circ}C$ for 12 h did not alleviate the decreased ability of these cells to form germ tubes in the single amino acid media (Table 18). Cells in mid log phase which were just regaining their ability to form germ tubes, did show an enhancement of inducibility by preincubation. Cells in late log phase, which formed germ tubes at normal levels, also demonstrated enhancement of germ tube formation by preincubation at $20^{\circ}C$.

Minimum Contact Time Required for Germ Tube Formation. In order to determine the minimum contact time required for initiation of germ tube formation in DMEM at $37^{\circ}C$, yeast cells were exposed to DMEM to various lengths of time, and transferred to fresh TMN buffer for a final 4 h incubation at $37^{\circ}C$ (Table 19). Very little germ tube formation occurred within the first 30 min incubation in DMEM or after the transfer of these cells to TMN buffer. After 35 min, 2% of the cells formed germ tubes during the DMEM exposure, while 16% more cells went on to form germ tube after the washing step and transfer to TMN buffer. By 40 min incubation, 15% of the cells had already formed germ tubes in DMEM, while 29% more cells formed germ tubes after the transfer. The experiment was not carried out further past 40 min exposure to DMEM since the majority of the cells

Table 18. Effect of TMN buffer preincubation on log phase cell germ tube inducibility.

Preincubation temperature (^{o}C)	Induction agent	Percent cells forming germ tube		
		Early log	Mid log	Late log
Control (no preincubation)	Arg	24	23	57
20	Arg	28	84	83
4	Arg	3	3	47
Control (no preincubation)	Gln	46	68	66
20	Gln	51	88	97
4	Gln	2	19	42

[a] *C. albicans* yeast cells were grown in SDB to various points in log phase, washed in TMN buffer, and incubated in TMN buffer (1.3 x 10^{6} cfu/ml) for 12 h. Cells were then inoculated into the media under standard conditions and incubated for 4 h at 37°C in the presence of arg or gln. Germ tube formation was then determined microscopically.

Table 19. Minimum contact time required for initiation of germ tube formation in DMEM at 37°C.[a]

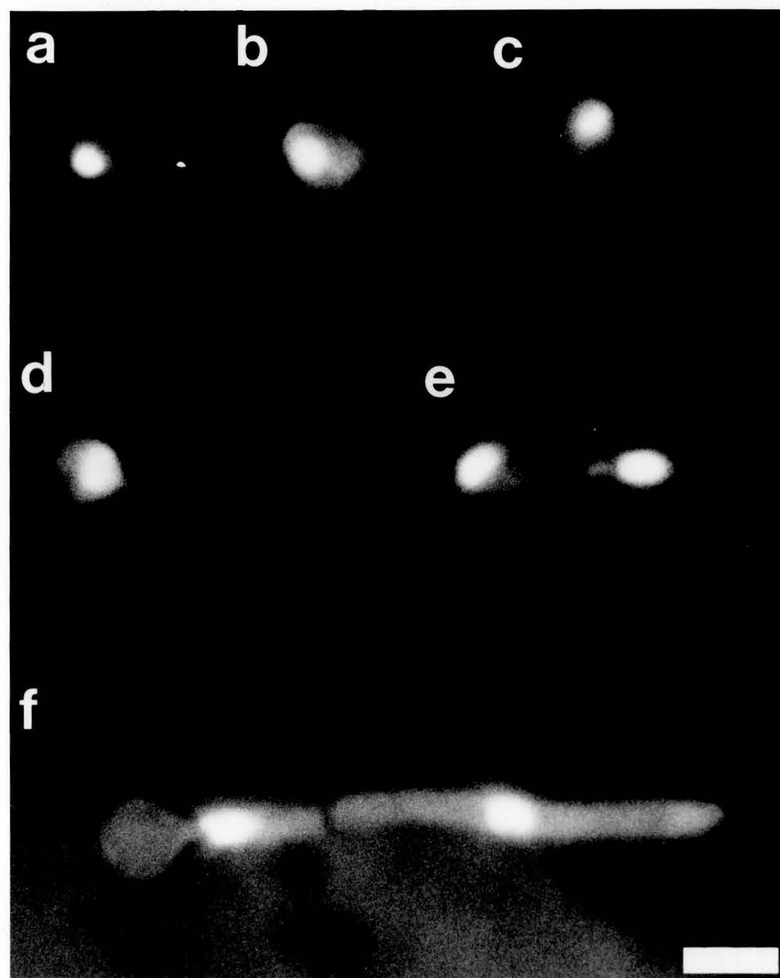
Time exposed to DMEM (min)	Percent cells forming germ tubes	
	Immediately after DMEM exposure	After 4 h additional incubation in buffer
0	0	2
5	0	0
10	0	1
15	0	3
20	0	2
25	0	4
30	0	4
35	0	18
40	15	44

[a] Washed stationary phase *C. albicans* yeast cells were exposed to DMEM at 37°C for various lengths of time, washed by millipore filtration with TMN buffer, and incubated in TMN buffer at 37°C for 4 h. Percent germ tubes was determined by counting 200 cells before and after the buffer incubation.

had already formed germ tubes during the exposure to DMEM. Yeast cells were also exposed to DMEM at 4°C for various lengths of time and transferred to TMN buffer at 37°C for 4 h. No germ tube formation was observed even after 1 h incubation in DMEM at 4°C, or after the TMN buffer incubation following the DMEM exposure. Therefore, in our system, the minimum contact time required for initiation of germ tube formation in DMEM of approximately 50% of the inoculum was 40 min incubation at 37°C.

Nuclear Staining of C. albicans yeast and hyphae. In order to follow the sequence of events of nuclear division and nuclear migration during gln induced germ tube formation, the elongating cells were stained with DAPI nuclear stain. Fig. 8a-8e shows cells stained after 0-4 h incubation in gln medium under standard conditions. By 4 h incubation, the length of the germ tube had reached 16 µm, was septated, and the parent cell and germ tube each contained a nucleus. As mentioned earlier, germ tubes in gln medium grew to a length of 10 to 25 µm and were almost never multicellular. A representative cell from a 3 h DMEM culture reached a length of 33 µm (Fig. 8f). The multicellular hypha showed a nucleus at the junction of the parent cell and the first germ tube compartment.

Fig. 8. Nuclear staining of C. albicans yeast and hyphae. Stationary phase C. albicans yeast cells were inoculated in gln medium or DMEM and incubated under standard conditions. At various intervals, samples were removed and the cells were stained with DAPI. Key: (a) Gln medium, 0 h; (b) Gln medium, 1 h; (c) Gln medium, 2 h; (d) Gln medium, 3 h; (e) Gln medium, 4 h; (f) DMEM, 3 h. Bar represents 5 microns.


a
b
c
d
e
f

DISCUSSION

The environmental and nutritional factors involved in transformation of _C. albicans_ ATCC 58716 yeast to the hyphal form were examined in this study. Initally, DMEM tissue culture medium was found to induce germ tube formation in nearly 100% of _C. albicans_ ATCC 58719 yeast cells (Table 2 and Fig. 2). Experiments in which individual amino acids were removed or combined clearly showed that arg and gln, two amino acids contained in DMEM, were primarily responsible for germ tube induction (Table 6). Addition of inorganic salts enhanced germ tube formation in the presence of either amino acid.

Germ tube formation in DMEM occurred over a narrow temperature range. Incubation at 37°C promoted complete germ tube formation as well as optimal germ tube lengths by 3 h incubation under standard conditions. Also, germ tube formation could occur at incubation temperatures at least as high as 42°C (107.6°F). These observations suggest that human body temperature may be conducive to germ tube formation under the correct conditions. Indeed, germ tube formation has been noted in vitro at 37°C in serum (Taschdjiam et al., 1960) and seminal plasma (Chattaway et al., 1976) as well as in sections of infected tissue from patients (Odds, 1979).

Other groups who studied temperature dependence had shown optimal germ tube formation at 37°C with decreasing percentages at temperatures above or below 37°C (Evans et al., 1975; Nishioka and Silva-Hunter, 1974; Simonetti et al., 1974). However, Hrmova and Drobnica (1981) observed germ tube formation at 28°C, noting that this contradicted statements by others concerning a strict requirement of 37°C incubation for germ tube formation. In their system, when stationary phase yeast cells were inoculated in aerated mineral medium enriched with vitamins and glucose, 57% of cells developed germ tubes approximately 10 µm in length after 4 h incubation at 28°C, although there was considerable strain variation. Obviously, groups who demonstrated germ tube formation over a range of of incubation temperatures had proven that there was not a strict requirement of 37°C incubation and that germ tube formation at incubation temperatures lower that 37°C was not uncommon. Even in our system, we observed 20% germ tube formation at 30°C incubation.

Incubation temperature may play a critical role in the regulation of protein synthesis necessary of initiation of germ tube formation. As suggested by Dabrowa and Howard (1984), the appearance of heat shock proteins in yeast cells after a temperature shift from 25°C to 37°C may indicate that certain proteins are produced by the cells only at the germ tube induction temperature. Heat shock

proteins, which are produced preferentially and occur in a wide variety of biological forms, are thought to be a defense mechanism against high temperatures or other adverse conditions. In this case, these proteins may be specifically involved in the germ tube induction process or in the germ tube elongation process. The control of genes important for germ tube formation may also be temperature dependent. If it were possible to obtain a mutant strain of C. albicans which differed from the parent strain only in its ability to form germ tubes at a normally non-permissive temperature (e.g. 25°C), studies might reveal temperature dependence of enzymes, protein synthesis, or gene activation in the parent strain. Bedell and Soll (1979) isolated a mutagenized strain of C. albicans which displayed 60% germ tube formation in Lee's medium at 25°C. However, germ tube formation took place only after 40 h incubation. It is possible that an alteration in the medium may have taken place after the extended incubation period and somehow promoted germ tube formation. The cells themselves may actually have been starved after depletion of nutrients during the extended incubation. Even the parent strain formed a small percentage of germ tubes.

One must keep in mind that temperature dependence is important in conjunction with the nutritional requirements for germ tube formation. For example, while incubation of C. albicans yeast cells in DMEM at 37°C promotes germ

tube formation, incubation in Sabouraud's Dextrose broth at 37°C results in budding.

The effect of pH on germ tube formation in our system was shown to be optimal over a certain pH range. In general, germ tube formation took place best at near neutral as well as at slightly acidic pH values. At pH values higher than pH 9, the decreased amount of germ tube formation might be explained by the fact that arg and gln change their molecular charge between pH 9 and pH 10 (pK_2 of arg = 9.09; pK_2 of gln = 9.13). This change in charge may somehow alter the germ tube inducing functions of the amino acids.

In *W. dermatiditis*, growth of the parasitic budding form occurs at near neutral pH values (5.5-6.5) (Szaniszlo et al., 1976). Growth of the multicellular form, the immediate precursor of the hyphal form, takes place only at lower pH levels (3.5-4.5). These observations suggested that the acidic culture conditions specifically inhibited bud emergence without inhibiting the events of growth, nuclear division, and cytokinesis.

Inoculum concentration in both DMEM and the single amino acid media was shown to be very critical. In DMEM, optimal germ tube formation took place at inoculum concentrations less than 1.3 x 10^6 cfu/ml (Fig. 4). At inoculum concentrations higher than this, there was strong evi-evidence suggesting that yeast cell replication was occur-

ring. Demonstration of an increase in the concentration of the cells in the sample, or an increase in the dry weight of the culture would provide additional supporting evidence. In the single amino acid media, budding did not appear to occur since, at higher inoculum concentrations, there was virtually no change in the percentage of budding cells (Fig. 6 and 7). If active budding was taking place, then the rate of budding would have to be constant to account for the constant proportion of budding cells over the inoculum range. Also, the cell concentration and dry weight of the culture would increase.

Why was there no germ tube formation at the higher inoculum concentrations? Since yeast cell replication probably took place in DMEM at higher inoculum concentrations, there may have been an inhibition of initiation of the cell's germ tube forming processes or a preferential initiation of the cell's budding processes. It is possible that the concentration of the germ tube inducing factor(s) per cell was too low. Yet, a preliminary experiment showed that DMEM could be diluted at least 256-fold without any decrease in the amount of germ tube formation (data not shown). In the case of arg (0.4 mM) and gln (4.0 mM) media, observable decreases in levels of germ tube formation occurred only at amino acid concentrations of 0.0004 mM or lower (Table 9). These observations suggest that the unavailability of the inducer was not responsible for the de-

creased germ tube formation.

Yeast cell replication at the higher inoculum concentrations may have been a response to an unfavorable environmental condition such as overcrowding. The close contact of the yeast cells after inoculation may have been a control signal which initiated budding rather than germ tube formation. The theory behind this is based on the mathematical calculation of the volume growth rate. Yeast cell generation time was approximately 2 h. A doubling in the number of yeast cells would correspond to a doubling in the volume of the culture taken up by the cells. By 8 h incubation, a constant rate of replication would have led to a 16-fold increase in volume taken up by the new cells. But if 100% germ tube formation in DMEM took place, a single yeast cell measuring 5 um would have formed a hypha 200 um in length thereby increasing the volume taken up by the cells 40-fold. This does not even take into account branching of the hyphae. In other words, the volume growth rate of hyphal forming cells would be greater than the volume growth rate of yeast forming cells. If one assumes that at a certain point, overcrowding of the cells prevented further replication, then this point would have been reached much sooner if the cells underwent hyphal formation. Therefore, at higher inoculum concentrations in DMEM, budding may have been the more efficient mode of replication. However, Herman and Soll (1984) actually noted

a similarilty in the rates of volume growth for buds and mycelium. Since the surface area of a mycelium with a volume growth rate similar to a bud is 27% greater than the surface area of the same bud, they proposed that, either mycelial cells must deposit 27% more cell wall material in the same amount of time as budding cells, or the wall of the newly forming mycelium must be composed of 27% less material. The fact that budding did not seem to take place in arg or gln media at the higher inoculum concentrations might also be explained by the fact that, nutritionally, the simple single amino acid media could be considered minimal media. The supportive nutrients in the media may have been exhausted very quickly and the cells remained metabolically inactive during the rest of the incubation.

It is also possible that the cells themselves produced an inhibitor of germ tube formation. Hazen and Cutler (1979) found that, in their tissue culture medium germ tube induction system, as the concentration of cells increased, the percent germ tube formation decreased. They demonstrated that the inhibition was not caused by nutrient depletion or by a change in the pH of the medium. When _C. albicans_ yeast cells were incubated under dialysis conditions, which theoretically would have removed an inhibitory substance, germ tube formation did occur at the higher inoculum concentrations. Their evidence indicated that _C. albicans_ produced a factor which they called a morphogenic

autoregulatory substance which was capable of influencing germ tube formation. The factor did not appear to inhibit replication since, at high inoculum concentrations, the cells continued to divide in the yeast phase (as determined by an increase in dry weight of the culture). Also, it was shown that the factor was not cysteine or phenethyl alcohol. Park (1984) studied the effect of population density on yeast-mycelial dimorphism in Aureobasidium pullulans. He also observed inhibition of hyphal formation at higher inoculum concentrations but had no evidence to suggest that the effect was caused by production and excretion into the medium of any substance by the metabolizing cells. The observed effect may have also resulted from the the removal by the cells of a substance required for hyphal induction.

When C. albicans yeast cells were inoculated in the single amino acid media under standard conditions and incubated at 37°C for 4 h, 100% germ tube formation was never achieved. We consistently observed that a certain subpopulation of inoculated cells never formed germ tubes. Yet, these cells had the potential to form germ tubes since 100% germ tube formation occurred in the DMEM control. Also, when inoculum cells were preincubated for 12 h at temperatures between 15°C and 25°C, we observed a significant increase in the percent germ tube formation in arg and gln media to a level comparable to that in the DMEM

control (Tables 14 and 15). This observation also indicated that each cell in the standard yeast cell inoculum did possess germ tube forming capability. What prevented this subpopulation from forming germ tubes under standard inoculation and incubation conditions?

One might question the viability of the cells inoculated into the single amino acid media. Though the viability of the cells was not tested by a standard procedure such as dye exclusion or microculture, one can assume 100% viability due to the fact that there was complete germ tube formation in the DMEM control.

The morphological profile of the non-responding cells indicated that this subpopulation consisted of both budding yeast cells as well as single yeast cells. Also, budding yeast cells could be seen to form germ tubes. In this case, the parent cell initially formed a germ tube and the daughter cell later formed a germ tube without having separated from the parent yeast cell. These observations suggest that non-responsiveness was not a characteristic of one certain morphological type.

Nuclear staining revealed that gln induced germ tube forming cells possessed normal nuclear division and nuclear migration. Cells which did not form germ tubes remained single cells with one nucleus.

It is possible that the amino acid concentration in our simple amino acid media was limiting. Using an induc-

tion system of 2.5 mM gln, Sullivan et al. (1983) had shown that all of the [U-^{14}C]glutamine had been removed from the medium and taken up by the cells after 4 h incubation at 37°C. In this study, germ tube formation in gln (4.0 mM) medium was never complete and did not significantly increase after 4 h incubation under standard conditions. If the gln in our sample has also been completely removed by 4 h, one could argue that the amino acid was no longer available for use by the uninduced cells. Yet, increasing the concentration of arg or gln tenfold did not cause an increase in germ tube formation (Table 8). Also, when these cells were incubated in a combination of arg and gln, there was no significant increase in germ tube formation (Table 11) suggesting that this subpopulation could not respond to either amino acid. Addition of glucose to either medium did not increase germ tube formation (Table 12).

This study as well as work done by other groups (Dabrowa and Howard, 1981; Mattia and Cassone, 1979; Soll and Herman, 1983) led us to believe that the non-responsiveness may have been associated with the metabolic state of the inoculum cells as well as to the nutritional complexity of the induction media.

As mentioned previously, preincubation of the inoculum in TMN buffer at temperatures between 15°C and 25°C significantly increased germ tube formation (Tables 14 and

15).

What effect did this preincubation have on the inoculum cells to enhance their germ tube forming capabilities? During the preincubation, there was no change in the morphological profile of the inoculum since the percentage of budded and single cells remained relatively constant. On the other hand, Soll and Herman (1983) noted that, in their system, after a 150 min starvation period in a buffered salts solution, the majority of budded cells cycled into the single cell population. Also, a germ tube inducing component was not released into the buffer since washing of the inoculum after preincubation did not prevent the increased germ tube formation upon inoculation into the single amino acid media.

Preincubation may have metabolically altered the inoculum cells. At the lower temperatures, the cells probably remained metabolically active. Since the buffer had little, if any, nutritional value, the cells would have had to rely on any internal stores or pools of energy sources. Hence, the buffer preincubation probably acted as a starvation period for the cells. Shepherd et al. (1980), using a glucose-glutamine induction medium also noted that, to obtain reproducible germ tube formation (approximately 90%), the inoculum cells (_C._ _albicans_ ATCC 10261) had to be aerated at $4^{o}C$ for 24 h in distilled water prior to inoculation. Sullivan et al. (1983) examined the intra-

cellular concentrations of certain metabolite pools to determine if they were significantly altered during starvation conditions which promoted the yeast to hyphae transition. After starvation conditions in phosphate buffer at $4^{o}C$ for 24 h, there was a significant decrease in intracellular glucose-6-phosphate while glycogen and trehalose stores remained relatively constant. When the starved cells were then incubated in GlcNAc induction medium at $28^{o}C$ (no germ tube formation) or $37^{o}C$ (germ tube formation, GlcNAc uptake was observed. But changes in the glucose-6-phosphate concentration, as well as in the trehalose concentration were similar at the two temperatures indicating that neither pool was a critical determinant in morphogenesis. Ironically, Schwartz and Larsh (1980) noted the absence of trehalose in the yeast form of C. albicans using the same assay method as Sullivan et al. (1983). Total amino acid pools were the same for starved yeast cells as wall as hyphal forming cells. Arg, gln, glutamate, and alanine accounted for 80% of the total amino acid pool. Interestingly, there was a decrease in the intracellular arg and gln concentrations after 3 h incubation in the GlcNAc germ tube induction medium. Sundaram et al. (1981), using the same starvation conditions, showed that the net synthesis of lipids occurred during starvation resulting in a doubling of the lipid content per cell. The lipid that was accumulated during starvation of

the yeast cells was rapidly utilized during germ tube formation. Significant lipid synthesis also occurs prior to hyphal formation in *Wangella dermatiditis*, the causative agent of phaeohyphomycosis (Calderone, 1976). The thin-walled yeast cell of this fungus can undergo conversion to the hyphal form only through an intermediary, stationary-phase, thick-walled cell. Transfer from 25°C to 37°C induces the conversion to the thick-walled cell, while the endogenous fatty acid concentration increases from 5.1% to 30%. It is thought that hyphal formation in *W. dermatiditis* may be dependent on this accumulation of fatty acid.

Since the aforementioned studies could not achieve complete germ tube formation in their induction systems without prior starvation, it is quite possible that changes in metabolite pools played an important role in the ability of cells to form germ tubes. Yet, in our system as well as as in other single amino acid induction systems, a large proportion of the inoculum could be induced to form germ tubes without starvation. Since the starved cells went on to nearly complete germ tube formation, it is assumed that the profile of metabolite levels for each starved cell was uniform and appropriate for initiation of germ tube formation. But if one were to study the metabolite pools of unstarved cells in our system, the measured metabolite levels would represent the average from the total population regardless of future cell responsiveness. It is pos-

sible that the metabolite levels from the non-starved non-responder cells might be different from those of non-starved responder cells. Though this information might shed light on the differences between the two types of cells, it would be virtually impossible to separate the non-starved inoculum cells into responder and non-responders prior to germ tube induction.

Another point mentioned earlier addressed the possibility that the nutritive complexity or richness of the induction medium might play an important role in responsiveness to inducers. Though we cannot eliminate the possibility that unresponsiveness to arg and gln was due to a metabolic "unreadiness" of the cells, the cells did respond to DMEM. In another type of experiment, it was shown that lag phase and early log phase cells exhibited a decreased ability to form germ tubes in the single amino acid media but not in DMEM (Fig. 7). Mattia and Cassone (1979) and Dabrowa and Howard (1981) both demonstrated that exponential phase cells could form germ tubes in a rich medium such as serum. Those investigators who observed a strict requirement of stationary phase yeast cells for germ tube formation used various simple amino acid or minimal media (Chaffin and Sogin, 1976; Hrmova and Drobnica, 1981; Soll and Bedell, 1978).

What role did arg and gln play in our system of germ tube induction? The answer to this question may possibly

be found in the study of the chitin synthesis pathway and the metabolism of the various DMEM amino acids. As shown in Fig. 9, gln is directly involved in the synthesis of chitin through the transfer of the amido group of gln to fructose-6-phosphate yielding glucosamine-6-phosphate. This reaction is catalized by the enzyme L-glutamine D-fructose-6-phosphate aminotransferase. Arg can be converted to gln through a four-step reaction, although the actual conversion was not shown. Likewise, pro, which was also to be an inducer in our system (Table 11), can also be converted to gln via a three-step reaction. Aspartate and asparagine, two other amino acids which could be converted to gln through multi-step reactions, induced germ tube formation approximately equal to that of arg and gln (data not shown). The twelve other DMEM amino acids which did not induce germ tube formation lacked the ability to be either converted to gln or to the product of gln that is the inducer. Three amino acids which participate in metabolic reactions yielding glutamate as a by-product (trytophan, tyrosine, and phenylalanine) did not promote germ tube formation even though glutamate can be converted to gln via glutamine synthetase. The data presented here suggest that, in our system, glutamine and related amino acids that can be converted either to glutamine or to a product of glutamine are responsible for germ tube induction.

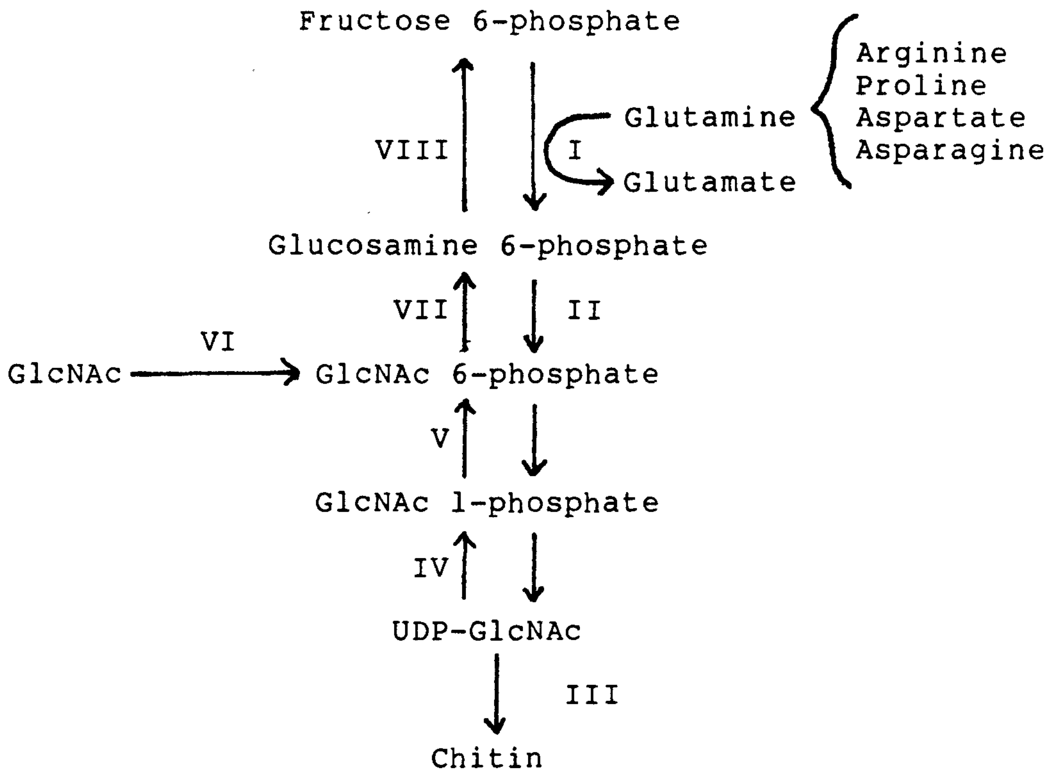


Fig. 9. The Chitin Synthesis Pathway. Enzymes: (I) L-glutamine D-fructose-6-phosphate aminotransferase; (II) glucosamine-phosphate acetyltransferase; (III) chitin synthase; (IV) UDP-N-acetylglucosamine pyrophosphorylase; (V) phosphoacetyl glucosamine mutase; (VI) N-acetylglucosamine kinase; (VII) N-acetylglucosamine-6-phosphate deacetylase; (VIII) glucosamine-6-phosphate deaminase.

Land et al. (1975) described substantial germ tube formation in their system induced by amino acids which entered metabolism through conversion to glutamate. They also noted that, when proline was the inducer and sole nitrogen source, normal proline metabolism was required for germ tube formation since chemical alteration of the active groups of proline prevented germ tube formation.

Dabrowa et al. (1976) observed that germ tube formation was induced by the amino acids entering metabolism via conversion to glutamate. Analogues of proline of the D-isomer markedly reduced germ tube formation in their system. Dabrowa and Howard (1981) later describe an active transport system for proline in _C. albicans_. Hyphal cells demonstrated a higher uptake rate than yeast cells, although this did not seem to be the result of an inducible system. Interestingly, the optimum pH and temperature for proline transport were pH 7.0 and 37°C, respectively. Addition of 16 other amino acids to the proline medium did not interfere with uptake of proline suggesting that the transport system was highly specific for proline.

Chiew et al. (1980) studied the L-glutamine D-fructose-6-phosphate aminotransferase in their 2.5 mM glutamine germ tube induction system. They found a direct correlation between the increase in specific activity of the enzyme and the yield of germ tube forming cells. The time of enzyme induction corresponded to the appearance of germ

tubes. Incubation of yeast cells in the gln medium increased the specific activity of the enzyme fourfold while incubation in GlcNAc medium only caused a 1.5-fold increase. As seen in Fig. 9, the entrance of GlcNAc into the chitin synthesis pathway bypasses the need for the aminotransferase and, hence, the small increase in the specific activity.

Work done by several investigators had been based on the theory that, since germ tube formation may be regarded as a cell wall morphogenesis, the control of the developmental process should be reflected in the regulation of the enzymes for cell wall polymer formation. GlcNAc, which induced greater than 90% germ tube formation from starved yeast cells, increased the enzymatic activity of chitin synthase 3-fold (Chiew et al., 1980). Braun and Calderone (1978) observed a 10-fold increase in the incorporation of GlcNAc into the hyphal cell wall, and a 2-fold increase in the activity of the membrane-bound chitin synthase. The enzyme exists as a proenzyme located along the plasma membrane. It is activated by a protease resulting in the laying down of chitin fibrils along the cell wall. They proposed that the proteolytic activator of chitin synthase binds more strongly to the enzyme of germ tube forming cells than to the yeast enzyme.

Shepherd et al. (1980) demonstrated that the maximum specific activity of GlcNAc kinase occurred 3 h after the

addition of GlcNAc. This corresponded to the time when all the GlcNAc had been removed from the medium. Beyond 3 h incubation, the enzyme activity decreased to baseline levels. Addition of more GlcNAc caused an increase in activity. N-acetylmannosamine (ManNAc) also induced the kinase, but probably as the result of sequential induction since ManNAc induced the synthesis of ManNAc-2-epimerase, the enzyme that catalyzes the conversion of ManNAc to GlcNAc. Germ tube formation also occurred in the presence of gln plus glucose, but the GlcNAc kinase was not induced. This indicated that gln and glucose entered the chitin synthesis pathway via a pathway that did not involve the non-phosphorylated GlcNAc. *De novo* synthesis of the kinase was required since inhibitors of protein and RNA synthesis inhibited the induction of the enzyme. In a similar induction system, Bhattacharya et al. (1974) also observed a halt in GlcNAc kinase synthesis upon removal of GlcNAc from the media, as well as an inhibition of induction of the enzyme by protein and RNA synthesis inhibitors.

Incubation of starved yeast cells with GlcNAc also induced the enzymes GlcNAc-6-phosphate deacetylase and Glc-6-phosphate deaminase (Gopal et al., 1982). The patterns of induction of these two enzymes were the same under conditions of germ tube formation (37°C) and where yeast cells metabolized GlcNAc with no change in morphology (25°C), indicating that these enzymes were not control

points in the dimorphic development of C. albicans.

The question as to whether arg and gln acted as triggers directly of via metabolism of the inducer has not been answered. A trigger in a bacterial spore model caused germination of the spore after a very brief (seconds) exposure to the trigger in a mutant that cannot further metabolize the amino acid suggesting that the inducing agent need not be metabolized. In our system, germ tube formation occurred only after the cells had been in contact with DMEM for at least 35 min at 37°C, implying, but not proving, that some metabolism of the inducing agent was necessary for germ tube formation. Dabrowa and Howard (1981) obtained similar results using a proline induction system. Cells which were incubated in the proline medium, washed at various intervals, and suspended in buffer, formed germ tubes only after 30 min incubation in the amino acid medium.

A second observation arguing against the idea of a direct triggering mechanism was the fact that when cells were exposed to DMEM at 4°C for 1 h, washed with TMN buffer, and then transferred to fresh buffer for 4 h incubation at 37°C, no germ tube formation took place. If a triggering mechanism was responsible for germ tube formation, then incubation in DMEM at 4°C would have led to germ tube formation since incubation at the low temperature probably would not have inhibited binding of the trigger to the

cell. However, incubation at 4°C would most likely prevent metabolism of most substances (inducers), thereby preventing germ tube induction. Incubation at the lower temperature may have also prevented transport of the inducer to the cell.

In contrast to those, Sullivan and Shepherd (1982) demonstrated that ManNAc could induce 50-60% germ tube formation in the presence of glucose or mannose. Yet, there was no apparent uptake or metabolism of ManNAc during the incubation period arguing against the theory of metabolism of the inducer. Further work by the same group using an immobilized GlcNAc as the inducer pointed to the involvement of a cell-surface receptor (Shepherd and Sullivan, 1983). Also, immobilized ManNAc induced approximately 70% germ tube formation, but only in the presence of glucose. Glucose probably acted as an energy source for basic metabolic functions such as glycoprotein synthesis and cell wall synthesis. They ultimately proposed that a morphogenic effector such as GlcNAc could bind to a cell-surface receptor and produce an intracellular message which primes the cell for morphogenesis.

Results of commitment time experiments (Table 19) sug-suggested that the inducers in DMEM need to be metabolized and do not act as signals themselves. In view of the recent data presented by Shepherd and Sullivan (1984), arg and gln may actually act as effectors. The incubation time

required for commitment may actually be needed for generation of the intracellular message. In our system, if arg and gln could be immobilized with agarose or a resin, they could be tested for germ tube induction in the presence and absence of glucose. If poly-arg or poly-gln could be synthesized, it would be interesting to test their ability to induce germ tube formation since they probably would be too large to enter the yeast cell. If they, too, could act as effectors, germ tube formation would probably take place in the presence of glucose. Also, it would be interesting to repeat the committment time experiments using free single amino acids and immobilized amino acids. If the single amino acids were actually acting as effectors, then there should be no difference in commitment times.

What could be the role of germ tube formation in the pathogenic process? In humans, _C. albicans_ is a normal resident of the gastrointestinal tract and its multiplication is thought to be controlled by the other normal resident bacterial flora. Under an adverse condition such as antibiotic or immunosuppresive therapy where there is a change in the normal bacterial flora, or during trauma when the yeast could be introduced to a new site (e.g. bloodstream), _C. albicans_ has the opportunity to proliferate. It is possible that elongation of the cell could facilitate colonization. As the germ tube elongates, branching occurs allowing the cell to extend to a new area. Logistically,

hyphal formation would cover more distance than budding in the same amount of time. Also, when a yeast cell enters a new environment, hyphal formation may allow the cell to explore the environment and determine what areas would be most suitable for colonization. The germ tube may also be a protective mechanism for C. albicans. Upon phagocytosis, the yeast cell can undergo germ tube formation. The elongating germ tube can puncture the white blood cell membrane thereby causing white blood cell death and allowing proliferation of the fungus.

Gow and Gooday (1984) proposed a model for germ tube formation and mycelial growth of C. albicans based on morphological and ultrastructural evidence. During hyphal formation, most of the cytoplasm of the parent yeast cell migrated into the elongating germ tube leaving behind an extensively vacuolated, phase light parent yeast cell. The parent yeast cell later became phase dark prior to formation of a second germ tube suggesting that the cytoplasm was regenerated. As the second germ tube emerged, the parent cell, again, became vacuolated. As hyphal elongation progressed, the apex of the germ tube remained phase dark suggesting that a "plug" of parent cell cytoplasm was migrating along the apex of the germ tube. Also, as the cytoplasm moved forward, a vacuolated hyphal compartment appeared. This compartment seemed to regenerate cytoplasm prior to any branching. They theorized that hyphal cell

wall was being synthesized by the cytoplasm and that the newly formed vacuolated hyphal compartments had to regenerate cytoplasmic components to support branch production. The linear growth of germ tubes and branches could be explained on the basis that the growth-supporting cytoplasm was a constant volume from the onset of germ tube formation.

Szaniszlo et al. (1983) placed emphasis on the control of cell wall sythesis in controlling dimorphism. Their views were based on the model for hyphal tip growth as proposed by Bartnicki-Garcia (1973). The model incorporates the idea that wall precursors, wall synthetic enzymes, and wall lytic enzymes are all present at the site of wall growth and that some or all of these components arrive at the sites compartmentalized in vesicles. Such vesicles are easily demonstrable in hyphal tips and developing buds by electron microscopy. They suggested that the most intriguing feature of dimorphism is not the question of how the fungal wall can come to possess different shapes, but how the fungal cell brings about site specific synthesis. They speculated on the presence of a polarization director, which is able to direct site specific wall synthesis and lysis in addition to numerous other functions, was responsible. Microtubules and microfilaments may fulfill this function. Interestingly, there is some evidence that cyclic AMP and divalent cations may have an effect on micro-

tubule assembly and function (Willingham and Paston, 1975).

San-Blas and San-Blas (1983) noted that, while morphogenesis is usally a part of the eucaryotic life cycle and essential to growth and maturation, fungal dimorphism is not essential to the fungal life cycle, but rather a response to a change in the habitat where it develops. They believe, though, that in order to elucidate the basic mechanisms involved in dimorphism of fungi, future research must be directed to the study of regulation at the genetic level since dimorphism must be primarily the result of selective gene activation.

In view of the fact that induction and activity of the enzymes involved in chitin synthesis are important for hyphal and yeast cell growth, future work in the area of antifungal therapy may concentrate on compounds which inhibit these enzymes.

It would be interesting to study the glutamine fructose-6-phosphate aminotransferase in our single amino acid system. If the activity of the enzyme increased in our system, this would be some positive evidence for the theory that arg and gln induce germ tube formation via their role in the synthesis of chitin. Also, one would not expect to see an increase in the activity of GlcNAc kinase.

Further work examining the arg, gln non-responding yeast cells may also shed light on the nature of the dimorphic event in C. albicans. Isolation of cells which were

shown to be non-responders would be critical. One possible method for isolating a non-responding cell would be by spreading a sample which had been incubated in media containing both arg and gln onto a SDA plate and removing a small section of agar which contained a single non-responder. This single yeast cell on SDA could then be incubated at 37°C for 24 h arising in a clone of a non-responder cell. Prepartation of arg gln agar plates may present an alternative method. It is possible that stationary phase yeast cells inoculated onto this agar may or may not form germ tubes and hyphae. Cells which do not form germ tubes would either remain single cells or would multiply by budding. Regardless, these non-responders could also be isolated and cloned for further testing. First responsiveness or non-responsiveness of the progeny to arg and gln would have to be determined. The possible outcomes would be total non-responsiveness, total responsiveness, or a mixture of both. If the progeny were non-responsive, starvation experiments might provide valuable information. Since starvation appeared to allow the non-responders to overcome their non-responsiveness, this could be confirmed by preincubation of the cloned cells in buffer and then testing for germ tube formation in arg and gln media. If they became responsive, the cloned cells could then be tested for any biochemical or metabolic differences before and after starvation. Also, differences in cytoplasmic

proteins might be observed.

The results of this study illustrate that dimorphism in *Candida albicans* is a complex process controlled by nutritional and environmental factors. Future work in this area will hopefully lead to a better understanding of the phenomenon of fungal dimorphism and, quite possibly, to a better understanding of growth and development of eucaryotic organisms.

SUMMARY

Nutritional and environmental factors affecting germ tube formation in the dimorphic, pathogenic fungus *Candida albicans* were investigated. Nearly 100% of stationary phase *Candida albicans* ATCC 58716 formed germ tubes when incubated at 37°C in Dulbecco's Modified Eagle's Medium (DMEM). Since DMEM is a chemically defined medium, elimination and reconstitution experiments utilizing the various components of the medium established that L-arginine (arg; 0.4 mM) and L-glutamine (gln; 4.0 mM), supplemented with certain inorganic salts, were responsible for germ tube induction in DMEM. In our induction system, either amino acid consistently induced 50-85% germ tube formation after 4 h incubation at 37°C. Percent germ tube formation as well as average germ tube length did not increase after 4 h incubation in arg or gln media.

Germ tube formation in DMEM was shown to be temperature dependent. Incubation of yeast cells at 37°C for 4 h provided maximum percent germ tube formation as well as maximum germ tube length (36.5 um). Inoculum concentration also appeared to be a critical factor in germ tube formation. Maximum germ tube formation in DMEM occurred when inoculum concentrations less than 1.3×10^6 cfu/ml were used, whereas maximum germ tube formation in the single

amino acid media took place at an inoculum concentration of 1.3 x 10^5 cfu/ml. Germ tube formation in DMEM was observed between pH 3 and pH 9 and in arg or gln media between pH 5 and pH 9. Also, inoculum cells in late lag or early log phase demonstrated a decreased ability to form germ tubes in arg of gln media, but not in DMEM.

Addition of glucose, vitamins, or other amino acids to the single amino acid media did not increase the percentage of germ tube formation. Combining arg and gln did not increase the percentage above that induced by either alone suggesting that a certain subpopulation of the inoculum was non-responsive to the inducers. Nuclear staining of gln-induced germ tubes revealed that normal nuclear division and migration usually occurred after germ tubes had emerged. The majority of non-responding yeast cells were single cells with one nucleus. Interestingly, preincubation of the stationary phase inoculum yeast cells in Tris-maleate-NaOH buffer pH 7 for 9 h at 20°C significantly increased the rate of subsequent germ tube formation in arg or gln media.

In this system, it was shown that arg and gln acted as inducers of germ tube formation although environmental factors such as pH, temperature, and inoculum size also affected this process. Based on the data obtained in this investigation and those reported in the literature, the role of the inducer in germ tube formation was discussed.

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

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

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