Isolation of a germination autoinhibitor produced by Candida albicans
by Kevin Clark Hazen

A thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in MICROBIOLOGY
Montana State University

Abstract:
Candida albicans is an important opportunistic pathogen of man. When this organism acts as a pathogen it characteristically forms yeast and mycelial cells, but as a saprophyte of the human host it forms only yeasts. Because of the possible relationship between morphology and pathogenesis, investigations were undertaken to determine how this organism regulates germination (yeast to mycelial conversion) . When attempts to induce germination were made, it was found that a low concentration of yeast cells was required. Cells produce a germination inhibitory factor which, after release into the surrounding medium, can affect yeast to mycelial conversion. A protocol to isolate the germination inhibitory factor was developed. This protocol involved charcoal adsorption, ether extraction, high performance liquid chromatography, and Sephadex LH-20 chromatography and resulted in a homogeneous compound. Preliminary identification of the germination inhibitor indicates that it may contain a heterocyclic constituent.

Other experiments to determine the effect of the germination inhibitor on cells were done. The germination inhibitor did not affect uptake of glucose or adenine by cells. However, uridine and amino acids were taken up less by cells exposed to the factor.

Various metals were tested to determine if they could affect the germination inhibitory factor. Only cobalt enhanced the germination inhibitory activity of the factor. Cobalt uptake by cells exposed to the germination inhibitory factor was increased over controls only if they were allowed to incubate in the presence of the factor for several hours before the addition of cobalt.

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Abstract

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Various metals were tested to determine if they could affect the germination inhibitory factor. Only cobalt enhanced the germination inhibitory activity of the factor. Cobalt uptake by cells exposed to the germination inhibitory factor was increased over controls only if they were allowed to incubate in the presence of the factor for several hours before the addition of cobalt.

INTRODUCTION

The most common disease of immunocompromised patients caused by yeasts is candidiasis (72). Species of *Candida*, most notably *Candida albicans* (Robin) Berkhout, have been reported to surpass *Cryptococcus neoformans* as the predominant agents of cerebral mycosis (118) and to be the most common yeasts to cause superficial infections in children (48).

The incidence of candidiasis, a general term to describe diseases caused by *Candida* species which range from mild and transient to life-threatening, has risen over the past three decades (64, 115). Several authors have suggested that this is because of an increased usage of antimicrobial drugs, immunosuppressive therapies, and prosthetic devices (2, 46, 64, 99, 115, 131, 169). However, because (superficial) candidiasis is also seen in apparently healthy patients, pathogenesis of the disease remains an enigma.

One aspect of candidiasis which has perhaps received the most attention concerns the dimorphic nature of *Candida albicans*, the most prevalent agent of the disease (46, 131). *C. albicans* cells can assume two *distinct* shapes: yeasts (ovoid, budding cells) and hyphae or

mycelia (tubular structures arising from either yeasts or other hyphae). Typical candidal lesions contain cells of both forms. When C. albicans is a saprophyte of the human host, it forms only yeasts. Because these observations are common, many investigators have presumed that the hyphal form of the organism is pathogenic while the yeast (Y) form is not and, thus, have not considered the importance of yeasts in development of disease (for review see 115). Indeed, although there are several studies which indicate that hyphal formation may be advantageous for the organism, vis a vis a mechanism of escape from phagocytic cells (38, 39, 84), there are also reports that only yeast form cells are required for pathogenesis (129, 166). Hence, if mycelial (M) formation is not required for pathogenesis, then it is at least a common result of host-parasite interactions.

Because elucidation of the mechanism(s) of dimorphic change of C. albicans may be potentially beneficial to man in terms of disease containment as well as providing insight into dimorphic mechanisms of other medically important fungi, such as Histoplasma capsulatum, Sporothrix schenckii, and Blastomyces dermatitidis, and because induction of morphological conversion of C.

albicans in vitro is relatively easy, a plethora of studies have been done on the phenomenon of yeast to mycelial (Y-->M) transition of C. albicans (for reviews see 12, 31, 54, 67, 96, 131, 132, 146). Most of the works prior to 1950 were concerned with the influence of the environment and nutritional factors on morphology. Physiological studies began in the late 1940s, and more recently, attempts have been made to understand the regulatory signals which dictate morphological change.

In spite of the number of studies done on Candida morphogenesis, the biochemical events important in initiation of a Y-->M transition have not been defined. In fact, evaluation of the literature in this area is a formidable task because of conflicting results and conclusions reported by many different research groups. One major problem seems to be that not all strains of C. albicans behave the same under similar conditons. The solution to this may not be simply to distribute standardized strains to various workers in the field because I have found that changes occur even within a single strain after repeated subculturing. Another problem is morphological interpretation. As alluded to earlier, Candida albicans has the ability to exist in many

morphological forms (59, 86, 115) such as budding yeasts, chains of yeasts, chains of elongated yeasts, germ tubes with a constricted base, germ tubes with a broad base and, not infrequently, a yeast cell will develop two germ tubes of which one is constricted and the other is broad based. To describe morphologically a Y-->M conversion at least five terms have been used which include hypha, germ tube, pseudohypha, pseudogerm tube, and filament--yet it is not always clear which morphology is actually observed by various investigators. Difficulty in interpreting and comparing various studies on C. albicans also stems from a lack of uniformity of growth conditions for obtaining yeast cells (see Table 1), various temperatures used to induce a Y-->M transition, a multitude of germination media used, and differences in incubation times used before cellular form is assessed.

These problems are best exemplified by studies on exogenous substances which affect morphogenesis of C. albicans. Table 1 lists some of the substances which when added to media prevent or promote M formation. Unfortunately, most of these reports have been of a phenomenological nature with little attempt at defining possible mechanisms of inhibition or stimulation of

Table 1. Factors which have been reported to affect Candida albicans Y-->M morphogenesis.

Factors tested	Temperature (°C) cells grown[a]	Temperature (°C) germination test	Resultant morphology[c]	Ref.
Carbohydrates				
glucose	37	37	M	76
glucose	37	40	NE	51
glucose	28	28	M,Y,ph	143
2-deoxyglucose	28	37	Y	142
polysaccharides	25	25	M	109
galactose	28	28	Y,ph	143
galactose	NS[d]	24	M	96
fructose	28	28	M,Y,ph	143
maltose	28	28	M,ph	143
sucrose	28	28	M,ph	143
GlcNac[e]	37	37	NE	158

Table 1 (cont'd).

Facters tested	Temperature (°C) cells grown	germinaiton test	Resultant morphology	Ref.
GlcNac	37	37	M	144
GlcNac	25	37	M	24
GlcNac	25	37	M	95
Metals				
Co	25	25	M	111
Co	28	37	NE	142
Zn	37	30	Y	168
Zn	NS	37	NE	157
Fe	37	37	M	78
Fe	NS	37	NE	157
Cu	NS	37	Y	157
Mn	37	37	M	144
Mg	NS	37	NE	157

Table 1 (cont'd).

Factors tested	Temperature (°C) cells grown	Temperature (°C) germination test	Resultant morphology	Ref.
Amino Acids				
cysteine	25	25	Y	101, 111
cysteine	28	28	Y	143
cysteine	30	30	NE	114
cysteine	37	37	NE	159
cysteine	NS	24	NE	96
hydroxyproline	NS	37	M	67
proline	37	37	M	35
proline	37	37	M	76
glutamate	30	30	M	114
glutamate	37	28	Y	92
methionine	37	28	M	92
arginine	37	37	M	76

Table 1 (cont'd).

Factors tested	Temperature (°C) cells grown	Temperature (°C) germination test	Resultant morphology	Ref.
alanine	37	37	M	76
Miscellaneous				
phenylethyl alcohol	28	37	Y	141
L-α-amino-n-butyric acid	37	37	M	93
acetate	28	28	Y,ph	143
lactate	28	28	Y,ph	143
NH_4Cl	37	37	Y	76
phosphate	37	37	Y	76
phosphate	37	37	Y	35
phosphate	NS	24	Y	96
phosphate	25	25	Y	109
phosphate	30	37	M	27
Tween 80	NS	37	M	136

Table 1 (cont'd)

Factors tested	Temperature (°C) cells grown	Temperature (°C) germination test	Resultant morphology	Ref.
Tween 80	28	37	NE	141
EDTA	25	25	M	104
cAMP	37	37	M	76
dibutyryl cAMP	30	37	M	29
dibutyryl cAMP	37	32-34	M	113
oxine + Co	25	25	Y	111
seminal fluid	30	37	M	28
low biotin conc.	37	30	M	167
antibody	RT[f]	37	Y	55
antibody	NS	37	M	147
Co-culture + bacteria	37	37	Y	3
Co-culture + bacteria	37	37	Y	4
Co-culture + bacteia	NS	37	Y	125

Table 1 (cont'd).

Factors tested	Temperature (°C) cells grown	Temperature (°C) germination test	Resultant morphology	Ref.
penicillin	25	25	M	109
aminopterin	25	25	M	109
5-fluorocytosine	28	37	Y	141
5-fluorocytosine	37	37	Y	121
nystatin	28	37	Y	141
2,4-dinitrophenol	28	37	Y	141
iodoacetamide	28	37	Y	141
chloramphenicol	28	37	NE	141
temperature	25	37	M	81
temperature	RT	37	M	89
temperature	NS	37	M	157
NaCl	37	37	Y	34

Table 1 (cont'd).

[a] Temperature at which cells were grown just prior to germination test.

[b] Temperature at which germination was induced.

[c] M=mycelial; Y=yeasts; ph=pseudohyphae; NE=no effect

[d] Not stated

[e] N-acetyl-D-glucoseamine

[f] Room temperature

morphogenesis. Indeed, the significance of this problem is manifested by contradictory reports concerning the effect of many different substances on Candida morphogenesis. For example, some workers obtained filamentous cells using high concentrations of glucose in an otherwise yeast inducing medium (76) but others found that glucose does not enhance germination (51, 109, 143). Reports have also indicated that glucose is important in maintaining the yeast form because if glucose levels are decreased (67) or if another carbon source such as galactose (96) or a less readily metabolizable carbohydrate is substituted for glucose (54, 109, 143) germination is enhanced.

Similar confusion exists when considering the effects of many other substances on morphogenesis. Until more definitive studies utilizing standardized conditions are done existing contradictions will not be resolved.

Regardless of the problems cited above some consistent results concerning various aspects of dimorphism of C. albicans have emerged from different laboratories.

Chemical analyses of Y and M cell walls and membranes have shown quantitative but not qualitative

differences between these morphological forms (25, 71). These findings are not unlike those obtained by others on Y and M of Mucor indicus (8) and Histoplasma capsulatum (73). Candida cell walls are composed chiefly of glucose as glucan, mannose as mannan (6), protein and a small amount of lipid (71). Whereas Y and M cell walls have a similar content of glucose and mannose, M cell walls have one third the amount of protein and three times the amount of glucosamine (presumably as chitin) as Y cell walls (25). The larger quantity of glucosamine in hyphal cell walls correlates with the suggestion (23) that an electron transparent layer which increases in width in the germ tube cell wall is composed of chitin. Increased chitin synthesis by M cells of C. albicans was also supported by others (18) who found that hyphal forming cells take up more N-acetyl-glucosamine than yeasts. In these studies, chitin deposition was found to occur at the site of budding for blastoconidial forming cells and at the apical region of hyphal forming cells.

Substantial differences in phospholipid and sterol content between cytoplasmic membranes of Y and M have been reported. Yeast cell membranes contain phosphotidyl serine, phosphotidyl inositol, sphingolipids, phosphotidyl

ethanolamine and phosphotidyl choline. However, only phosphotidyl ethanolamine and phosphotidyl choline have been detected in membranes of exponential phase M cells (94). This correlates well with other observations (5) that the major lipids synthesized from ^{14}C-acetate during Y-->M conversion are phosphotidyl choline, phosphotidyl ethanolamine, and neutral lipids. Overall lipid synthesis, however, is lower in M than in Y cells (152).

Considering the evidence that only stationary phase yeast cells are capable of germinating (24, 148), it is interesting that stationary phase yeast cell membranes more closely resemble membranes of exponential phase mycelial cells that exponential phase yeast cells (70). Taken a step further, these data may imply that changes in the chemical make-up of the cell membrane mark the initiation of morphogenesis.

At the ultrastructural level, differences between yeasts and young hyphae have been observed. Yeast cell walls seem to be composed of five layers whereas germ tubes have only four layers (23, 137). However, hyphae associated with invasion of human tissues may be composed of six distinct cell wall layers (126). There is disagreement as to which of the five layers of yeast cell

wall disappear during hyphal development. Cassone, Simonetti, and Strippoli (23) feel that dissolution the second layer of the germinating yeast cell occurs during hyphal initiation, but Scherwitz, Martin, and Ueberberg (137) believe the third layer disappears. It is interesting that initiation of bud and germ tube formation appear similar in that the major part of emerging daughter cell walls of both forms are composed of material contiguous with the fourth layer of the mother cell (137). This may indicate that the fourth cell wall layer is composed of flexible material.

In addition to fewer cell wall layers usually found in hyphal cells the wall of this form of C. albicans is about half the thickness of a yeast cell wall (23). Similar correlations between yeast and mycelial cell walls have been observed in Histoplasma capsulatum (40) and in Mucor (8).

As alluded to above, chitin synthesis rates appear to be different between Y and M cells. The key enzyme in the production of chitin from glucose, L-glutamine-D-fructose-6-phosphate aminotransferase, has been demonstrated to have a higher specific activity in germinating cells. In addition, induction of the enzyme

appears to be related to the appearance of germ tubes (30).

Further evidence that chitin synthesis is involved in germination of C. albicans cells are the observations that the last enzyme in the chitin synthesis pathway, chitin synthetase (synthase), has a higher specific activity in hyphal cells and hyphal cells incorporate ten times more N-acetyglucosamine (GlcNAc) than yeasts (18). Modeling their studies on those done on Saccharomyces cerevisiae (21, 41, 42, 69), Braun and Calderone (18) suggested that the proteolytic activator of chitin synthetase binds more strongly to the enzyme of mycelial cells than to the enzyme of yeast cells.

GlcNAc, a precursor for chitin synthesis, has been demonstrated to induce germ tube formation (144). Although the observation has been disputed (158) it is difficult to evaluate the controversy because of differences in experimental protocol. GlcNAc has also been shown to induce germination more quickly (24) and to induce Y-->M conversion in cells which normally do not germinate (i.e., early and mid-logarithmic phase cells) (95).

Exogenous GlcNAc appears to induce synthesis of a

chitin synthesis enzyme N-acetylglucosamine kinase by C. albicans (13). However, the induction kinetics are similar in both yeast and mycelial cells (142) as are the specific activities. This suggests that N-acetylglucosamine kinase is not important in Y-->M conversion (142) which is not surprising because the organism can synthesize chitin from glucose.

The possible involvement of carbohydrate utilization pathways in morphological changes of C. albicans cells has been investigated. In studies using metabolic inhibitors, Hasilik(57) reported that phosphofructokinase (PFK) and glucose-6-phosphate dehydrogenase (G6PD) are important for growth. Chattaway et al. (26) studied some enzymes of glycolysis, of the oxidative pentose pathway, and the first enzyme of chitin synthesis, viz., L-glutamine-D-fructose-6-phosphate aminotransferase. PFK activity was lowest when germination was at its peak, and cells which had germinated contained less PFK activity than yeast form cells. The investigators felt that the low PFK activity would allow more fructose-6-phosphate to be available for chitin synthesis via L-glutamine-D-fructose-6-phosphate aminotransferase. This idea correlates with their earlier findings that chitin is more abundant in mycelial form

cells than in yeasts (25). Unfortunately, the mycelial extracts used for enzyme assays were obtained from cells grown at 40°C and yeast extracts were from cells grown at 30°C. Therefore, it is impossible to know if reduced PFK levels are required for Y-->M morphogenesis or if the reduction in activity is due to an increase in incubation temperature.

Adenosine-3':5'-cyclic monophosphate (cAMP), which is well established as being involved in various metabolic events of prokaryotes (130) as well as morphogenesis of Mucor racemosus (119) has been studied for its participation in Y-->M conversion of C. albicans. Exogenous addition of cAMP to C. albicans has been shown to decrease RNA and protein synthesis (14) and to increase Y-->M conversion (77). Although others found that cAMP would not promote M development (113) Y-->M transition was enhanced by addition of the lipophilic dibutyryl derivative of cAMP (29, 113).

Attempts to correlate intracellular cAMP levels with germination reveal that the cAMP concentration increases prior to (113) and during germination (29) but these changes also correlate with a shifting of the incubation temperature from 30°C to 37°C. However, in accordance

with correlating cAMP changes with morphogenesis, substances which either increase or decrease intracellular cAMP levels increased or decreased germination, respectively (29).

Only one investigation has endeavored to integrate cAMP levels with metabolism. The addition of glucose to the C. albicans growth medium did not affect levels of cAMP or GlcNAc catabolic enzymes. Glucose, on the other hand, did repress the enzymes and cAMP levels in Saccharomyces cerevisiae. These data suggest the C. albicans catabolic repression mechanisms differ from other fungi (145). Furthermore, the mechanisms by which increased glucose may prevent mycelial formation may not involve cAMP. Reduced oxygen tension or increased atmospheric CO_2 have been shown, in other studies, to favor Y-->M conversion by Candida albicans (54, 67, 92, 96, 146, 163). Under anaerobic conditions (100% H_2 atmosphere, however, the percentage of germinating cells in serum was reduced (36). Although attempts bave been make to correlate repressed respiratory activity (mitochondrial repression) with germ tube formation (76, 77) the data obtained from using various respiratory inhibitors are not clear. A recent paper contains data

indicating that respiration rate is not different between Y cells and cells undergoing Y-->M conversion (62). However, the tubular structures observed by the investigators appear to be elongated buds and not true germ tubes.

It is interesting to note that under conditions which are considered to enhance filamentation, viz., reduced glucose concentrations (see earlier discussion), the respiratory pathway that becomes dominant is cyanide resistant. Under less limiting conditions, the major respiratory pathway is cyanide sensitive (74). Clearly, more work is needed in this area to understand how O_2 tension relates to morphogenesis.

Protein synthesis occurs at similar rates in Y and M forms of C. albicans (33). Whereas cytoplasmic protein synthesis is required for morphogenesis, mitochondrial protein synthesis seems unnecessary (141).

In an attempt to determine whether there are proteins unique to yeast or mycelial cells, Dabrowa et al.(33) used disc gel electrophoresis to analyze cell free extracts of these forms. Two protein bands were reported to be unique to yeasts and one band unique to mycelial cells. However, with two-dimensional gel electrophoresis

mycelial cells were found to have fewer proteins than yeast cells and proteins unique to mycelial forms were not detected (19, 20, 89, 90, 91). It was suggested that Y-->M conversion occurs because of post-translational protein modifications (90, 91) or differential gene expression (19).

The relationship between nucleic acid synthesis and Candida morphogenesis is not clear. Recently, evidence has been produced which indicates that germination may be a sexual characteristic of C. albicans (117). However, this view is not universally accepted (122, 135).

During a Y-->M conversion nuclear division occurs when the germ tube reaches a critical length (10, 151), whereas in budding cells nuclear division occurs when the bud attains a critical volume (10, 150). Production of M form cells was found to occur in the presence of an inhibitor of purine synthesis (109) and in adenine deficient strains of C. albicans (75). However, a Y-->M transition was blocked by using other inhibitors of RNA synthesis (141) and M form cells were reported as having more RNA than the yeast form (168). Then again, others found DNA and RNA synthesis to be similar in Y and M forms (33, 159,). It may be more informative to evaluate RNA

(33, 159,). It may be more informative to evaluate RNA synthesis in terms of which species of RNA is synthesized during initiation of morphogenesis.

All of the studies mentioned above have yielded important and, in some cases, significant insights into the nature of the mechanism of morphological change in C. albicans. However, two "classical" sets of investigations on C. albicans dimorphism which are relatively ignored by current authors but which are the original impetus for many contemporary studies are those of Walter J. Nickerson and those concerning the production of morphological regulatory substances.

Nickerson conducted extensive investigations into the the role of cysteine in Y-->M conversion of Candida albicans because of several observations. First, Trichophyton rubrum was found to release an -SH containing compound which inhibited germination of C. albicans (66, 108). Second, 10^{-2} M cysteine inhibited germination of wild type and of a filamentous mutant of C. albicans (102, 111). Third, C. albicans cells accumulated cobalt when cobalt was present in the growth medium (112), and it was known that cysteine chelates cobaltous ions (1). Fourth, the presence of a readily utilizable carbon source

such as glucose did not prevent Y-->M morphogenesis (109). From these observations, Nickerson postulated that filamentation resulted when cell growth occurs without cell division (101, 102) and cell division occurs only in the presence of a sufficient number of -SH groups (provided, for instance, by cysteine) (102). In support of this concept, C. albicans was described as having an NADH dependent cystine reductase (110, 132) of which the NADH is provided through glucose metabolism (109). Hence, carbon sources such as polysaccharides, would not yield enough NADH to power the cystine reductase and, thus, a Y-->M conversion would result.

In related studies a filamentous mutant of C. albicans, designated strain 806, was induced to grow as a yeast when incubated in the presence of a substance released from wild-type cells (105). Furthermore, strain 806 and wild-type mycelial cells, but not yeasts, released protons into the medium surrounding the colony (104). Nickerson (104) proposed that release of H^+ indicated that they were unavailable for reducing the appropriate substance(s) required for maintenance of yeast growth (103). Although the final proton acceptor molecule in yeasts was not identified, it was suggested that it may

reside in the cell wall (52, 106, 107). Furthermore, by accepting protons and subsequently forming more covalent bonds (possibly as -SH groups) the acceptor molecule in the wall would favor the prolate spheroid shape of the yeast form (53).

As in other investigations on C. albicans Y-->M conversion, however, the observations of Nickerson and co-workers suffer because the studies were done on developed yeast and mycelial cells rather than on events which occur prior to morphological evidence of a transition. Therefore, it is not possible to make definitive conclusions about the importance of cysteine in initiating Candida morphogenesis.

In 1939, Langeron and Guerra (80) reported that C. albicans cells located on apposed sides of two parallel streaks would not germinate when the distance between the streaks was sufficiently short. These workers believed that the effect might be due to production by C. albicans of a germination inhibitory factor, but some felt that this so called "Langeron effect" was merely due to nutritional depletion (88). Others, however, sought to determine the nature of the germination inhibitor of which two substances, phenylethyl alcohol (PEA) and tryptophol,

were isolated (83). Because these substances inhibited growth of C. albicans it is difficult to evaluate their effects on morphogenesis.

It is particularly interesting that other investigators have made similar observations to those of Langeron and Guerra (80), viz., that C. albicans produces substances, presumably other than tryptophol and PEA, which affect morphogenesis (66, 124, 134). The identity of these substances or how they metabolicallly affect a Y-->M conversion is unknown. The production of germination self-inhibitors by C. albicans yeast cells provides an explanation for the long observed phenomenon in the clinical laboratory. That is, germination will not occur during a germ tube test if the inoculum of the C. albicans yeast cells is too concentrated (68, 79, 143).

It is not unreasonable to expect that C. albicans does produce a germination self-inhibitor. Several other fungi such as Syncephalastrum racemosum (63), Glomerella cingulatta (82), and Mucor racemosus (100) and others (11, 15, 47, 87, 155), have been shown to produce such substances. If a germination self-inhibitor of C. albicans can be identified, then its utilization as a tool to understanding dimorphism of the organism is potentially

limitless. Furthermore, information gained from such studies could provide a much needed basis for treatment approaches to combat candidiasis.

It has been my approach to determine if, in fact, C. albicans does produce a germination self-inhibitor. Once this was established I have attempted to formulate an isolation scheme which would yield a homogeneous preparation for the autoinhibitor and allow preliminary chemical characterization to be done.

Also, in some preliminary experiments which involved using a crude preparation of the germination self-inhibitor, the metabolic effect of the inhibitor on C. albicans cells was determined.

MATERIALS AND METHODS

Organisms

Candida albicans 9938 (Mycology Unit, Tulane Medical School, New Orleans, LA) was used in all experiments unless otherwise indicated. *C. albicans* strains 2252, 3153, and 550 were obtained as gifts from T.G. Mitchell (Duke Universtiy, Durham, NC) and strains 804a and 394 from Richard R. Morrison (University of Oklahoma, Norman, OK). *Candida parapsilosis*, *Candida tropicalis*, *Crytococcus laurentii* C-16, and *Saccharomyces cerevisiae* were from the mycologic culture collection at Montana State Universtiy. All organisms were maintained on corn meal agar slants and transferred every six months.

Cultural Conditions and Preparation of Organisms for Germination Tests

Unless stated otherwise, fungi were grown in 50 ml of glucose (2.0%)-yeast extract (0.3%)-peptone(1.0%) (GYEP) broth contained in 250 ml Erlenmeyer flasks at 37°C for 48 h under constant aeration by rotation at 160 rpm (Incubator Shaker, New Brunswick Scientific, Edison, NJ). Under these conditions, over 99% of *C. albicans* remained in the yeast form. The yeasts were used immediately after incubation or within a 24 h postincubation period during

which they were refrigerated. Before use in germination experiments, the cells were washed three times with 0.15 M NaCl or sterile, deionized water.

Treatment of Glassware

All glassware was washed with 7X detergent (Flow Laboratories, Inglewood, CA) in distilled water and rinsed with deionized water. Sterilization was by autoclaving.

In some experiments glassware, washed as above, was acid cleaned by soaking for 30 min at 60°C in nitric acid (previously diluted by mixing one part concentrated nitric acid with seven parts deionized water), rinsing repetitively with deionized water, covering with aluminum foil, and sterilizing by dry heat at 400°C for 4 h. All acid cleaned glassware was used within 24 h of treatment.

Germination Media

Fetal calf serum (FCS) (Microbiological Associates, Walkersville, MD), tissue culture medium (TC199) (Microbiological Associates), and two synthetic media, derived from TC199, and designated GM-1 and GM-2 were used. When media were supplemented with TC199, one ml of a 10X concentrated tissue culture medium (10X TC199)

(Grand Island Biological, Grand Island, NY) was added per 9 ml of test medium. Otherwise, TC199 containing 1.4 g of $NaHCO_3$ / liter and 25 mM N-2-hydroxymethyl piperazine-N'-2-ethanesulfonic acid buffer (Microbiological Associates) was utilized.

GM-1 and GM-2 were prepared from 10X stock solutions (Table 2) by adding 1 part stock to 7 parts sterile, deionized water and 2 parts sterile, 0.05 M, pH 7.4 (at 37°C) tris (hydroxymethyl)aminomethane hydrochloride buffer (pH 7.4 Tris buffer).

Preparation of Cell Filtrates Containing a Germination Autoregulatory Substance

Cell filtrates were prepared by inoculating either TC199 or GM-1 with 2×10^8 (3 times washed) C. albicans 9938 cells per ml of medium and incubating for 15 h to 18 h at 37°C in sterile, glass centrifuge tubes or 1000 ml graduated cylinders. When TC199 was used, incubation was in 5% CO_2 : 95% air. After incubation, suspensions were centrifuged and the supernatant liquid was collected, filtered through a 0.45 um porosity membrane, and adjusted to pH 7.2 by dropwise addition of 0.1 N NaOH (when TC199 was used) or to pH 7.4 by dropwise addition of tris (hyroxymethyl)aminoethane base (Trizma base, Sigma). All

Table 2. Formulations for 10X concentrated GM-1 and GM-2.

Medium	Subtance[a]	Amount(mg/l)	Molarity(M)
GM-1 and GM-2	folic acid[b]	10	2.27×10^{-5}
	L-arginine HCl	1266	6.01×10^{-3}
	L-methionine	150	1.01×10^{-3}
	L-proline	692	6.01×10^{-3}
	L-histidine HCl	420	2.00×10^{-3}
GM-1	glucose	1000	5.56×10^{-3}
GM-2	glucose	5000	2.78×10^{-2}
	d-biotin	10	4.09×10^{-5}
	NH_4SO_4	250	1.89×10^{-3}
	$NaH_2PO_4 \cdot H_2O$	413	2.99×10^{-3}
	$Na_2HPO_4 \cdot 12H_2O$	717	2.00×10^{-3}

[a] All chemicals are products of Sigma Chemical Company (St. Louis, MO) except glucose and NH_4SO_4 (J.T. Baker Chemical Co., Phillipsburg, N.J.).

[b] Components were heated into solution, and the final solution was sterilized by filtration through 0.45 um porosity membranes (HAWP, Millipore Corp., Bedford, MA)

cell filtrates were used within 48 h during which they were refrigerated.

Germination Assays

Tube germination assay. 5×10^5 washed *C. albicans* cells were resuspended in 1 ml of an appropriate medium and placed into a glass tube (12 x 75 mm) and incubated for 3 h at 37°C in air when FCS was used or in 5% CO_2 : 95% air (to prevent a drop in pH) when TC199 was used. Cell morphologies were determined at the end of the incubation period.

Diffusion chamber germination assay. To test the effect of a population of yeast cells on germination by anotner population of cells, plastic chambers consisting of an upper and a bottom well (Blind Well Chambers with a 5/16 inch [ca. 6.35 mm] bottom well, and Bio-Rad Laboratories, Richmond, CA), separated by a 0.45 um porosity, detergent free membrane (HATF, Millipore Corp., Bedford, MA) were used (Figure 1). Each well received an equal volume of yeast cell suspension. Chambers were incubated for 3 h at 37°C before the morphologies of the cells in each chamber were assessed. When the suspending medium was TC199, incubation was in 5% CO_2 : 95% air.

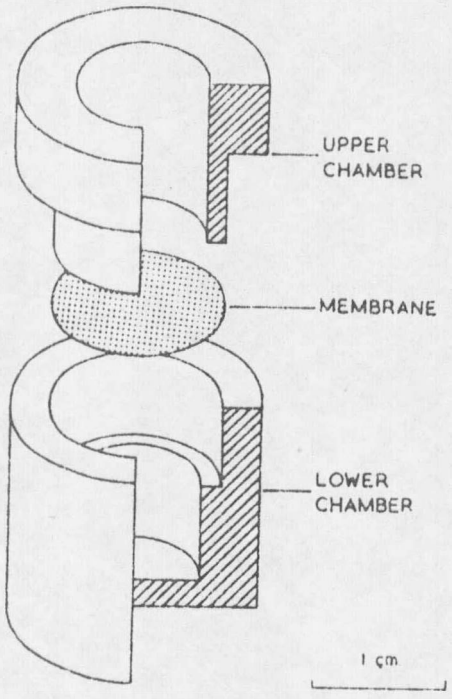


Figure 1. Schematic diagram of the chamber used in the diffusion chamber germination assay (from 37).

Broth culture germination assay. Eight milliliters of medium was placed in a 10 ml Erlenmeyer flask and warmed to 37°C prior to additon of yeasts. Eight microliters of a yeast cell suspension containing 5×10^7-1×10^8 cells/ml was added to each flask to give a starting cell concentration of 5×10^4-1×10^5 cells/ml.

Cells were incubated for 4 h (unless otherwise noted) at 37°C and rotated at 160 rpm. Cellular morphologies were determined after the desired period of incubation.

Assessment of Morphology

Under conditions which favor germination of yeast cells, C. albicans may develop many different shapes. For convenience these morphologies were separated into three categories: germ tubes; pseudohyphae; and yeasts (Figure 2). A germ tube is defined as an appendage, borne from a mother cell, which is at least two times greater in length than the mother cell and the lateral walls are parallel throughout the length of the appendage. Yeasts are considered as those cells which have an ovoid shape and are composed of no more than three contiguous cells. All units of contiguous cells not fitting these two categories are designated pseudohyphae.

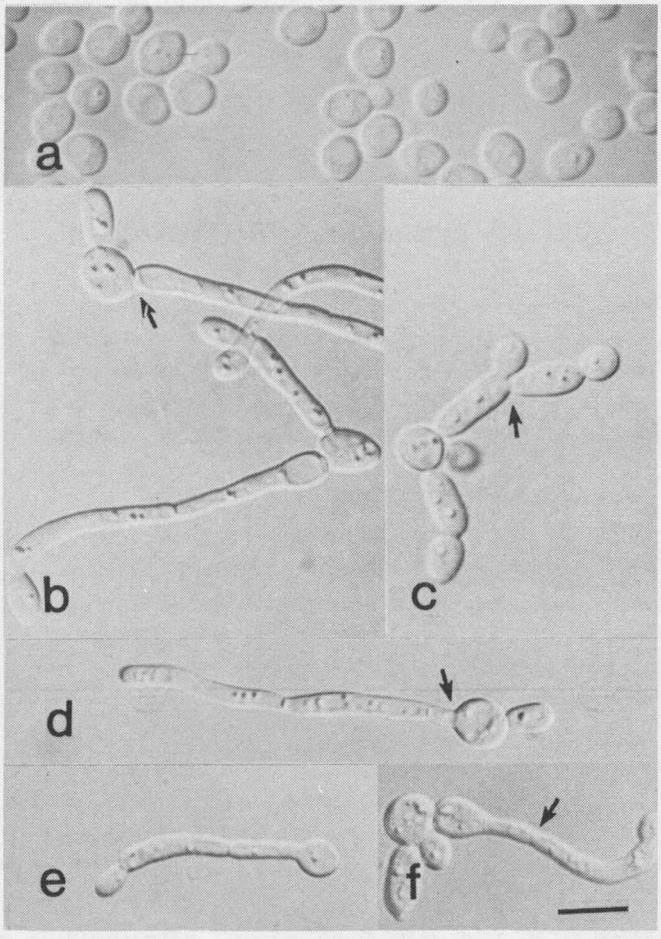


Figure 2. Different morphologies of C. albicans: a) yeasts; b) pseudohypha (arrow indicates point of constriction); c) pseudohyphae (arrow indicates point of constriction); d) germ tube (arrow indicates lack of basal constriction). Frames e and f display two forms which are difficult to categorize (arrow in f indicates point of constriction along tube). Bar represents 5 um.

Colorimetric Test for Free Sulfhydral Groups

A sodium nitroprusside (Sigma) reagent was prepared by the method of Block at al. (16). In the presence of sufficient free sulfhydral groups, the reagent shifts from light brown to lavender. The addition of three drops of reagent to 0.9 ml of test solution detects cysteine to a minimum concentration of 1 mM.

Anion Exchange Chromatography

Dowex-1, 200 mesh, anion exchange resin (Sigma) was converted and maintained in the acetate form (Schroeder, 1967). Columns (0.7 by 8 cm) were prepared and equilibrated at 5°C with 0.05 M tris (hydroxymethyl)-aminomethanehydrochloride buffer in 0.05 M NaCl at pH 8.26 (at 4°C) (pH 8.26 Tris buffer). Ten milliliters of medium (TC199 or culture filtrate prepared in TC199) were lyophilized and rehydrated with 0.9 ml of pH 8.26 Tris buffer. The rehydrated material was washed through the column with the buffer until 10 ml of eluate was collected. Nitroprusside testing was done on the medium before and after passsage through the anion-exchange resin. The eluate was also nutritionally supplemented by adding 0.5 ml of 10X TC199 and tested against C.albicans

9938 in the tube germination assay.

Cysteine, Tryptophol, and Phenylethyl Alcohol (PEA)

Freshly prepared cysteine HCl (Fischer Scientific Co., Fair Lawn, NJ) solutions of various concentrations were tested for germination inhibitory activity by placing 0.1 ml of cysteine solution in 0.9 ml of TC199 and running the tube germination assay. The presence of detectable sulfhydrals in each solution was determined by the nitroprusside test.

Tryptophol and PEA (Sigma) were tested with the tube assay by adding to TC199 the amount of material which would yield the concentration (40 and 20 ug/ml, respectively) determined by Lingappa, et al (83) to be inhibitory to germination as well as five times and twenty-five times these concentrations. Also, growth of C. albicans 9938 over an 8 h incubaton period at 37°C in the presence of twenty-five times concentration of each substance was evaluated by direct counting on a hemacytometer.

Gas chromatography was used to determine the presence of PEA in a cell filtrate. One microliter samples were injected into a 10% Carbowax 20 M column (2 m x 3 m) at

190°C with helium at 15 ml/min as the carrier. Various concentrations of PEA were prepared in TC199 and in cell filtrate. To each of these standard concentration of PEA, a respectively similar concentration of cyclohexane (as an internal standard) was added.

The presence of tryptophol in active cell filtrates was also tested by gas chromatography. A 1 ul volume of the appropriate sample was injected into a column (2 m x 3 m) of 2% OV-101 at 150°C using helium at 20 ml/min as the carrier. Standard concentrations of tryptophol were prepared in TC199 and in cell filtrates.

Determination of Cell Growth

C. albicans 9938 cells were incubated in either cell filtrate or TC199 in glass tubes (12 x 75 mm) for 8 h at 37°C in 5% CO_2. Direct counts and colony forming units were determined at 0, 3, and 8 h after incubation by, respectively, counting on a hemacytometer and plating dilutions of the cell suspension on Sabouraud dextrose agar containing 50 U of penicillin and 50 ug of streptomycin per ml.

Preparation of Metal Stock Solutions

All metal stock solutions were prepared in deionized

water and were sterilized by either filtration through 0.45 um porosity membranes (HAWP, Millipore) or autoclaving. Unless otherwise noted, reagent grade metal compounds were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ). One molar stock solutions of the following metal compounds were prepared: $CuCl_2 \cdot 2H_2O$; $CaCl_2$; $ZnCl_2$; $CoCl_2 \cdot 6H_2O$; $NiCl_2 \cdot 6H_2O$; $MnCl_2 \cdot 4H_2O$; $MgCl_2 \cdot 6H_2O$; and $Co(NO_3)_2 \cdot 6H_2O$. Stock solutions of $Fe(NO_3)_3 \cdot 9H_2O$ (Sigma) and $FeCl_3 \cdot 6H_2O$ were 10^{-1} M, and stock solutions of MoO_3 (Mallinckrodt Chemical Works, St. Louis, MO) were prepared at 10^{-3} and 10^{-4} M. Further dillutions, when required, of any of the stock solutions were performed by transferring with sterile, plastic, pipettes the appropriate volume of metal solution to diluent (deionized water). All metal solutions were stored in glass containers at 4°C.

Addition of Metal to Germination Media

Whenever possible, 8 ul of the appropriate solution of metal was added to 8 ml of medium in the broth germination assay. Hence, metal solutions were diluted 1:1000 for testing. Some metal stock solutions had to be diluted 1:100 for testing because they were insufficiently

soluble to make concentrated solutions which could be diluted 1:1000. In such cases, 80 ul of a metal solution was added to 7.92 ml of medium.

Assay for Testing Metal Enhancement of Germination Autoregulatory Activity

The percent inhibition of germination by a cell filtrate with or without added metal was determined by comparison with germination levels of cells in GM-2 with or without added metal, respectively, by the equation:

$$\% \text{ inhib.} = 100 - \frac{\%\text{germ. in cell filtrate (plus metal)}}{\%\text{germ. in culture medium (plus metal)}} \times 100$$

All experiments were done at least twice and each metal was tested in triplicate culture in each experiment.

^{60}Co and ^{65}Zn Uptake Experiments

To determine the effect of cell filtrate on the ability of *C. albicans* cells and other yeasts to take up Co and Zn, 1 uCi of $^{60}CoCl$ (sp. act. 44.7 mCi/mg) or $^{65}ZnCl_2$ (sp. act. 1.1 mCi/mg) (New England Nuclear. Boston, MA) were added to each 8 ml of medium, which had been supplemented with 10^{-5} M $CoCl_2 \cdot 6H_2O$ or 10^{-6} M $ZnCl_2$, respectively, in the broth culture assay. The volume of ^{60}Co or ^{65}Zn solution which was added to each 8 ml of

medium never exceeded 25 ul.

The number of detectable emissions per minute associated with the cells in each flask at the end of an incubation period was determined by filtering cell suspensions through separate, pre-weighed, 13 mm, 0.45 um porosity membranes (HAWP, Millipore), washing the membranes with at least 10 ml of distilled H_2O, and subjecting the membranes to emission spectroscopy (Biogamma Counter, Beckman Instruments, Inc., Fullerton, CA). In all instances the counts associated with cells were several orders of magnitude above background. Each filter was then dried by heating at 55°C for at least 4 h and weighed. The cpm/mg dry weight of cells for each flask was calculated by the formula:

$$\frac{\text{cpm of } {}^{60}\text{Co (or } {}^{65}\text{Zn)}}{\text{mg dry weight of cells}} =$$

$$\frac{\text{cpm}_{\text{cells}} - \text{cpm}_{\text{background}}}{\text{wt (mg) of membrane plus cells} - \text{wt (mg) of filter}}$$

Determination of Cobalt and Zinc Levels in Media

Cobalt and zinc concentration in GM-1 and cell filtrate, which was made with GM-1, was determined by (method 289.1 for Zn and method 219.2 for Co in Methods for Chemical Analysis of Water and Wastes, Environmental Protecton Agency, Cincinnati, OH, 1979) and

performed by the Chemistry Analytical Station at Montana State University.

Uptake of Metabolites Experiments

To determine the effect of cell filtrate on uptake of various substrates by C. albicans, the broth culture germination assay was used. Each 8 ml broth was supplemented with approximately 1.6 uCi of D-[^{14}C(U)]-glucose (sp. act. 45.9 Ci/mmlo), 1.6 uCi of [2-^{3}H]-adenine (sp. act. 24 Ci/mmol), or 2 uCi of the combination of L-[ring 2, side chain 2,3-^{3}H]-histidine, L-[methyl-^{3}H]-methionine, L-[2,3-^{3}H]-arginine, and L-[^{3}H(G)]-proline at the beginning of the incubation period. All radiochemicals were purchased from New England Nuclear Corp. (Boston, MA) except [2-^{3}H]-adenine (Amersham Corp., Arlington Heights, IL.). For each time period that uptake determinations were made, triplicate cultures per medium tested were run. At 30 min and 4 h, cultures were placed into ice water baths. Morphologies were assessed, and the cpm associated with cells from each flask were determined by filtering 2 ml and 4 ml aliquots of cell suspensions through individual 2.5 cm glass fiber filters (GF/C, Whatman Laboratory Products, Inc., Clifton, NJ), washing

the filters with 10 ml of distilled H_2O, drying the filters at 60°C for at least 4 h, and subjecting the filters to scintillation spectroscopy (Tri-Carb 460 CD Liquid Scintillation System, Packard Instruments, Downers Grove, IL). The scintillator solution was composed of 5.0 g of 2,5-diphenyloxazole (PPO, Research Products International Corp., Mt. Prospect, IL.) per 1000 ml of toluene.

The dry weight of cells from each 2 ml or 4 ml aliquot was determined by extrapolaton from the mean dry weight of cells in three 80 ml broths of similar medium, each of which were contained in 125 Erlenmeyer flasks, inoculated with the same inoculum size and incubated under identical conditions as the 8 ml broths. The 80 ml broths were not supplemented with any radiochemical. To obtain the total dry weight of cells in each 80 ml broth, cell suspensions were filtered through pre-weighed, 2.5 cm, 0.45 um porosity membranes (HAWP, Millipore), dried, weighed, and the difference calculated. This method was used because 2 and 4 ml cell suspensions would not yield a detectable weight of cells. It was considered a reasonable approximation of the cell dry weight in the 8 ml broths because the surface area to volume ratio was

similar in both types of broth cultures (8 and 80 ml) and the morphology of the cells in each type of broth was similar after identical periods of incubation.

The average cpm/mg dry weight of cells was calculated with the following equation:

$$\frac{\text{avg. cpm of radiolabelled metabolite}}{\text{mg dry weight of cells}} = \frac{\bar{X}\text{cpm}_{\text{cells(2 or 4 ml)}} - \bar{X}\text{cpm}_{\text{background}}}{\bar{X}\text{wt (mg) cells (2 or 4 ml)}}$$

Incorporation of each radiochemical was determined by precipitation of cellular material onto filters (HAWP, Millipore) with 0°C, 25% trichloroacetic acid (TCA). For these experiments all radiochemicals mentioned above were used, except a ^{14}C-[U]-amino acids mixture (New England Nuclear) was substituted for ^{3}H-amino acids. Also, cells were exposed to TC199 or cell filtrate made in TC199 and the tube germination assay was used. The effect of cell filtrate on incorporation of radiolabelled substrate was determined by comparing the ratio of the TCA treatment for cells in cell filtrate to the similar ratio for cells in TC199.

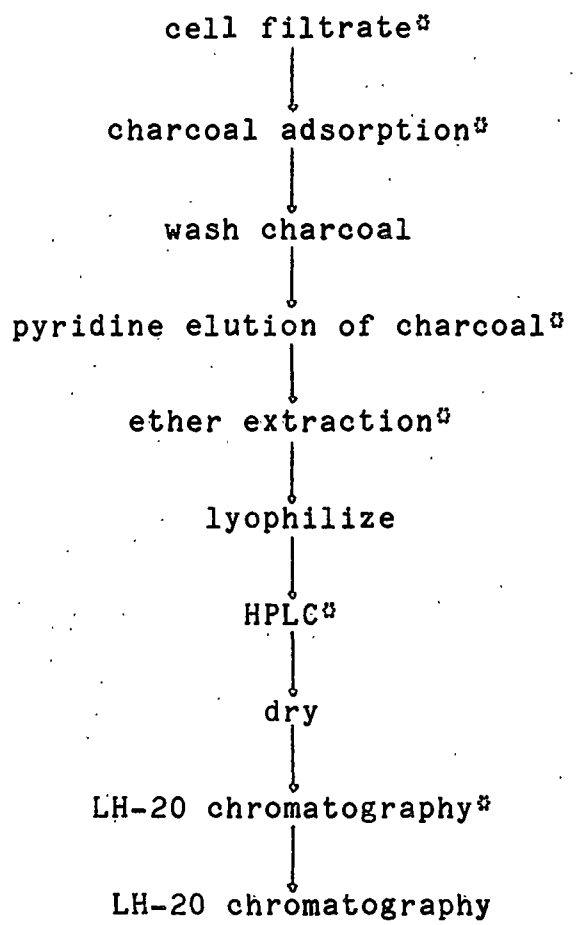
Isolation Procedure of Germination Inhibitory Substance

The procedure for isolating a germination inhibitory substance of C. albicans was obtained by modification of the method of Sakagami et al. (133).

Pretreatment of activated charcoal. By filtration, 50 g of activated charcoal was treated with the following materials in succession: 300 ml of deionized water; 300 ml of 3% disodium ethylenediaminetetraacetic acid (EDTA); 300 ml of concentrated acetic acid; 500 ml of pyridine; and 300 ml of deionized water. The treated charcoal was dried by heating at 50°C overnight.

Isolation. A flow diagram for the isolation procedure and points at which fractions were tested for germination inhibitory activity are presented in Figure 3. Treated charcoal (1 g/l) was added to cell filtrate made in GM-1, and the suspension was constantly stirred for 3 h at room temperature. The charcoal was pelleted by centrifugation (2200 x g, 45 min) and washed two times with deionized water. The charcoal pellets were resuspended in pyridine (approximately 20 ml/g dry weight of charcoal) and shaken at 160 rpm at 37°C for one to two hours. The pyridine was separated from the charcoal by filtration through sintered

Figure 3. Flow diagram for isolation of the germination autoregulatory substance and points at which activity of fractions were tested.



* Indicates that fractions were tested for germination inhibitory activity by the broth culture assay.

glass and then through a glass fiber membrane (GF/C, Whatman). The filtrate was flash evaporated at 45°C. To promote evaporation of pyridine from the resultant dried material, it was three times resolubilized with ether saturated water (ESW) and flash evaporated.

The final dried material was resolubilized in approximately 60 ml of ESW and extracted three times with an equal volume of diethylether (ether). The combined ether phases were dried by flash evaporation at 45°C, resolubilized with 20 to 30 ml of ESW and lyophilized.

The lyophilized material was resolubilized with 1.0 ml of methanol (Baker Instra-Analyzed Methanol, J.T. Baker Chem. Co.) per 1000 ml of original cell filtrate fraction. The solution was cooled in an ice water bath and clarified by centrifugation (400 x g) for 10 min. The material in the pellet did not inhibit germination of C. albicans 394 cells. The supernatant fluid was subjected to high pressure liquid chromatography (HPLC).

HPLC was done with a Beckman model 332 gradient liquid chromatograph equipped with a 250 ul injection loop, a 254 nm UV absorbance detector, and a 25 cm x 4.6 mm I.D. reverse phase column (Ultrasphere-ODS, Beckman Instruments, Inc., Fullerton, CA).

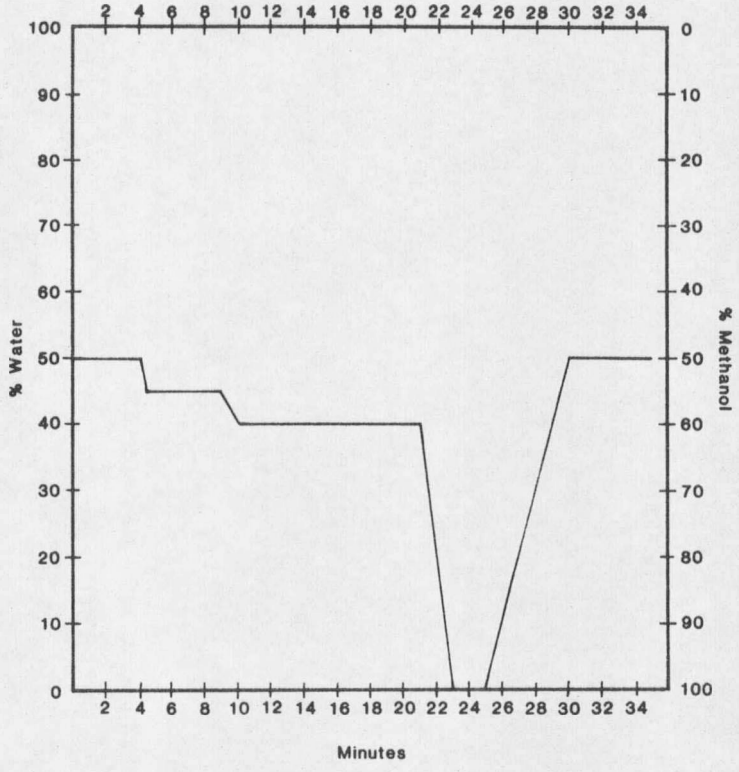


Figure 4. HPLC solvent program used for fractionation of ether extract.

The solvent program (Figure 4) involved a step gradient from 50% methanol in water to 100% methanol.

Two hundred and fifty microliter aliquots were injected and eluate fractions were collected. Each fraction was flash evaporated (45°C) and lyophilized.

The portion of HPLC eluate which contained the germination inhibitory factor was further fractionated by LH-20 chromatography (Pharmacia Fine Chemicals, Uppsula, Sweden). Bed dimensions were 1.5 x 31 cm, and the solvent was 90% methanol in 0.05 M ammonium acetate. Eluate fractions were flash evaporated and lyopholized.

As alluded to earlier, at various points during the isolation procedure fractions were tested for germination inhibitory activity. To do this, fractions were lyophilized and then resolubilized with deionized water or ESW (ether was removed by bubbling N_2 through the solution) to give, upon dilution into GM-2, a final concentration from one to twenty-five times that of the original cell filtrate. The broth culture germination assay was used to test the fractions. C. albicans 394 was used for germination tests.

Determination of Purity

Homogeneity of the material in the final fraction containing germination inhibitory activity was determined by thin layer chromatography (TLC). Five solvent systems were used for TLC (155):

1. n-butanol: acetic acid: water (4:1:1, v/v/v)
2. n-butanol
3. petroleum ether: diethylether: acetic acid (90:10:1, v/v/v)
4. ethanol (95%): NH_3 (25%) (4:1, v/v)
5. benzene: methanol: acetic acid (45:8:8, v/v/v)

Silica Gel IB and Silica Gel IF (J.T. Baker Co.) were used as sorbents and were heated to 110°C for 30 min prior to use.

Detection was done by determining lack of fluorescence on a normally fluorescent plate (Silica Gel IF) or by spraying plates with ninhydrin or chromate sulfuric acid and heating at 100°C for 5 to 15 minutes (151).

Proton Magnetic Resonance (PMR) Spectroscopy

PMR spectroscopy was performed on a Bruker WM-250 250 MHz Multinuclear Fourier Transform NMR Spectrometer by Dr. John Cardellina of the Department of Chemistry at Montana

State University. To prepare a fraction for analysis, lyophilized material was solubilized in a small volume (between 0.5 and 1.0 ml) of d_6-dimethylsulfoxide (d_6-DMSO, Sigma). For PMR spectroscopy of the final LH-20 fraction, the lyophilized material was twice solubilized with D_2O (99.8%D, Sigma) and lyophilized before finally solubilizing with d_6-DMSO. Chemical shifts are expressed as δ units downfield from trimethyl-silane (TMS, $\delta = 0$).

RESULTS

Effect of Cell Concentration on Germination

Different yeast cell concentrations of C. albicans 9938 were tested for germination in TC199 and FCS. As seen in Figure 5, the lower the concentration of yeast cells in the inoculum, the higher the percentage of cells which germinate. In subsequent experiments, a starting cell concentration for germination assays was 5×10^5 cells/ml unless otherwise stated.

Effect of Cell Growth Phase on Germination

Cells were inoculated into GYEP and incubated for 56 h. At each hour up to 37 h, an aliquot of cells was harvested, washed, and tested (with the tube germination assay) for germinative ability. Cell concentration in the GYEP broths was also determined at each hour by hemacytometer counts and colony counts on Sabouraud dextrose agar. Stationary phase cells yielded the highest percentage of cells which germinated (Figure 6). Germinative potential appears to be a cyclic event because the percentage of germinating cells increased and decreased during the twenty hours after reaching stationary phase (11 h). After 31 h the germinative

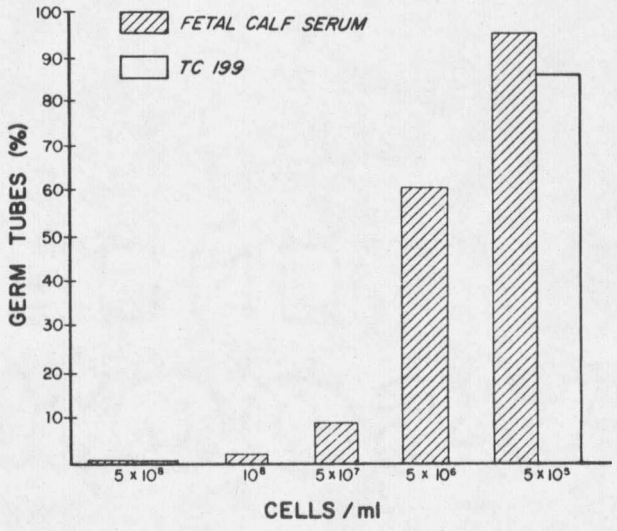


Figure 5. Effect of cell concentration on germ tube formation. Germination was conducted with the tube germination assay. The results are the mean of four experiments, each run in duplicate. At each cell concentration tested, the standard error of the mean for germination was not more than ± 4%.

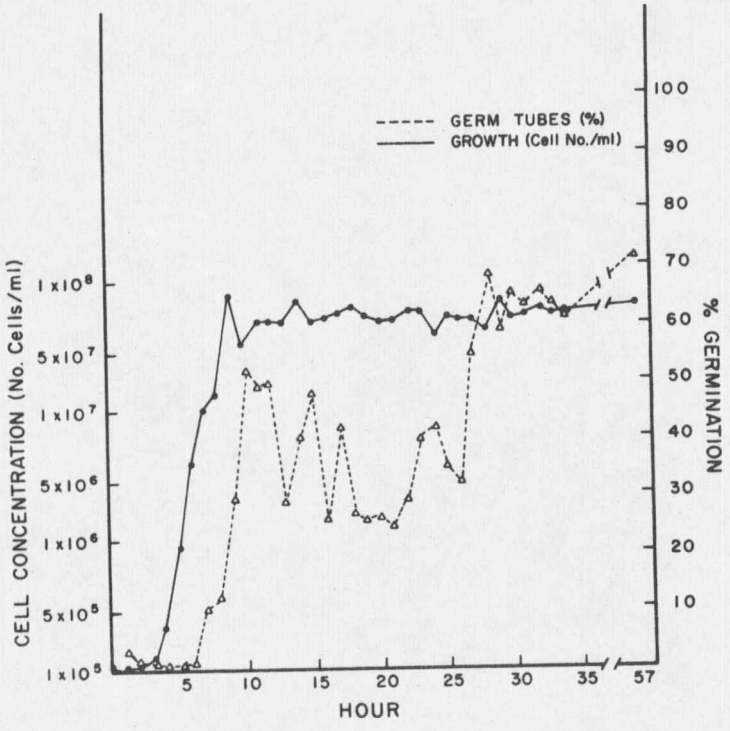


Figure 6. Effect of growth phase on germination. Cells were inoculated in GYEP and allowed to grow for 57 h. At each hour up to 35 h and at 57 h cell concentrations in the GYEP broths were determined and the ability of cells to germinate was tested with the tube germination assay. The results shown are from one experiment. The experiment was run several times and similar results were obtained.

capacity of cells appears to remain stable. Exponential phase cells showed increased ability to germinate as they approached early stationary phase. These data suggest that production of germination machinery is an ongoing process in a growing cell population.

Effect of a High Concentration of *C. albicans* Yeast Cells on a Low Concentration of Yeast Cells

When a low concentration of cells 5×10^5 cells/ml, in either FCS or TC199 was placed in the lower well of a diffusion chamber and 1×10^8 yeast cells/ml of the same medium was placed in the top well, germination by the cells in the lower well was markedly decreased (Table 3). If a low concentration of cells was placed in the top well, germination by the cells in the lower well was not affected. These data indicate that a high cell concentration may release a soluble factor that inhibits germination.

Kinetics of Production of Germination Regulator

Cell suspensions composed of washed 2×10^8 *C. albicans* 9934 yeasts/ml of 0.01 M pH 7.4 Tris buffer were placed in 50 ml centrifuge tubes and incubated for 24 h at 37°C. Every 3 h, cell filtrate from one centrifuge tube

Table 3. Effect of a high concentration (10^8 cells/ml) of various yeasts on germination by C. albicans.

Concn of cells in top well (cells/ml)	Organism	Results in bottom compartment after incubation					
		Germ tubes (%)		Pseudo-germ tubes (%)		Yeasts (%)	
		FCS[b]	TC199	FCS	TC199	FCS	TC199
1×10^4	*C. albicans* 9938	47.8 (5.4)[c]	32.9 (21.0)	10.5 (2.1)	9.5 (7.3)	41.9 (3.9)	57.7 (5.4)
5×10^5	*C. albicans* 9938	70.1 (3.8)	72.8 (7.3)	9.1 (2.1)	12.7 (4.3)	21.4 (3.7)	14.6 (5.4)
1×10^8	*C. laurentii*	71.6 (2.5)	ND[d]	8.6 (1.5)	ND	20.1 (4.1)	ND
1×10^8	*C. parapsilosis*	71.5 (5.1)	ND	6.8 (4.1)	ND	21.8 (3.8)	ND

[a] A suspension containing 5×10^5 *C. albicans* 9938 yeast cells per ml was placed in the bottom well of a two-compartment chamber, and suspensions containing the indicated concentration of yeasts was placed in the upper well. The wells were separated by a 0.45-μm membrane (Millipore). Results were determined after 3 h of incubation.

[b] In some experiments the fungal cells were suspended in FCS while tissue medium (TC199) was used in other instances.

[c] Standard deviation of the mean of at least two separate experiments run in duplicate each time.

[d] ND, Not done.

was harvested and stored in a refrigerator. After the 24 h incubation period ended, the filtrates were tested for germination inhibitory activity. At 0 h, no inhibitory activity was present and by 9 h activity was greatest (Figure 7). Twenty-four hour cell filtrate contained significantly less activity than 9 h cell filtrates.

These data suggest that production of the germination inhibitor requires an active process.

Because 15-18 h cell filtrates strongly inhibited germination and for convenience, cell filtrates were usually harvested at this time.

Effect of Cell Filtrates of Different Yeasts on *C. albicans* 9938

To determine if *C. albicans* 9938 as well as other yeasts produce a soluble factor which inhibits germination of *C. albicans*, yeast cell suspensions of 2×10^8 cells/ml of TC199 were incubated for 15 h at 37°C. The suspensions were filtered, and the cell filtrates were pH adjusted and tested either directly after harvesting or after nutritional restoration with 10X TC199. Similar amounts of germination of *C. albicans* occurred with cell filtrates of *C. albicans* 9938, *C. albicans* 2252, and *C. tropicalis*. Cell filtrates from *C. laurentii* and *C. parapsilosis* did

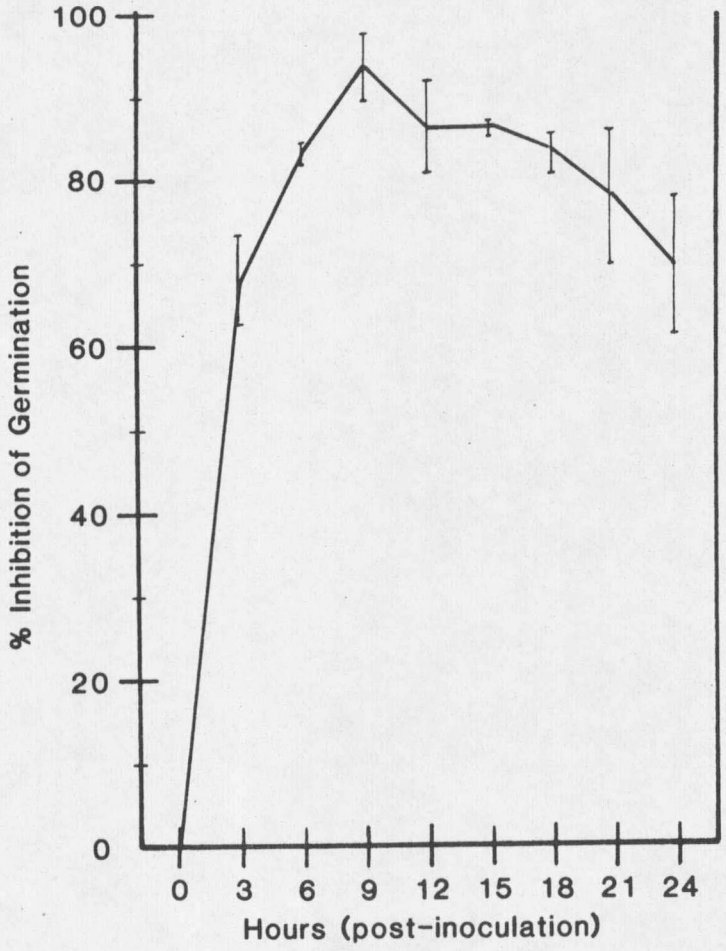


Figure 7. Effect of amount of incubation on production of germination inhibitor. Suspensions of 2 x 10^8 cells/ml of 0.01 M pH 7.4 Tris buffer were placed in 50 ml centrifuge tubes and incubated at 37°C for up to 24 h. At each time period, duplicate 50 ml suspensions were centrifuged and the supernatant fluid was sterilized by filtration. The filtrates were tested for germination inhibitory activity with the broth culture assay. In all assays, cells in control medium yielded > 85% germinated forms.

not, however, affect germination. In all cases, the same results were obtained with nutritionally unsupplemented and supplemented cell filtrates. Furthermore, in the diffusion chamber assay, a high yeast cell density (10^8 cells/ml) of either one of the latter two yeasts in the upper well did not affect germination of 5×10^5 *C. albicans* 9938 cells/ml in the lower well (Table 3). The absence of the production of a germination regulator by *C. parapsilosis* and *C. laurentii* does not appear to be due to inhibition of metabolism because both organisms were found to grow well in TC199.

Effect of Nutrition on Germination

Because it was possible that cell filtrates lack a sufficient concentration of nutrients to support germination, they were supplemented with 10X TC199 to give concentrations of either 1X or 2X TC199. Nutritionally supplemented cell filtrates were tested against unsupplemented cell filtrates for capacity to support germination of *C. albicans* 9938 cells with the tube germination assay.

The germination percentages of cells in cell filtrates with and without nutritional reconstitution were 52.2 and 55.9, respectively.

The percentage of cells germinating in 1X TC199 and 2X TC199 was 86.0%. An additional control showed that 2X TC199 does not support enhanced germination of cells at a high yeast cell concentration (2×10^8 cells/ml).

Effect of Dialysis on Germination Activity

The germination capacity of 10^8 yeasts/ml of TC199 was improved when the suspension was placed in a dialysis bag and incubated under conditions favoring dialysis, indicating that a soluble factor which inhibits germination was removed. By 5 h of incubation, approximately 20% more yeasts had germinated under conditions of dialysis than non-dialyzed yeast cell suspensions (Table 4). The increased percent germ tube formation correlated with a decrease in percent yeast cells after germination. Dialysis had no effect on the germination capacity of a low density (5×10^5 cells/ml) of yeast cells.

Effect of Cysteine, Tryptophol, and PEA on Germination

Because cysteine has been reported to inhibit germination by *C. albicans* cells (111) various concentrations of cysteine were prepared in TC199 and tested on *C. albicans* 9938 cells with the tube germination

Table 4. Effect of dialysis on germination.

Concn of cells (cells/ml)[a]	Solvent[b]	Results after incubation		
		Germ tubes (%)[c]	Pseudo-germ tubes (%)	Yeasts (%)
1×10^8	+	36.1 (8.2)[d]	6.8 (2.5)	57.1 (7.0)
1×10^8	−	14.8 (6.2)	3.3 (1.8)	81.9 (5.6)
5×10^5	+	90.4 (6.0)	3.3 (1.4)	6.3 (6.3)
5×10^5	−	84.1 (6.5)	8.4 (3.6)	7.5 (3.4)

[a] The indicated suspensions of *C. albicans* 9938 were made in TC199, and 1.0 ml of the suspension was placed in the dialysis bag.

[b] +, Dialysis bags were submerged in 50 volumes of TC199 per volume inside the bag during incubation; −, dialysis bags were incubated in an empty beaker. To prevent desiccation during incubation, these experiments were run in a humidified incubator.

[c] Suspensions containing 1×10^8 yeasts per ml were incubated for 5 h, while suspensions containing 5×10^5 yeasts per ml were incubated for 3 h.

[d] Standard deviation of the mean of at least two separate experiments run in duplicate each time.

assay. Reduction of germination was only minimal at concentrations of 1 mM cysteine (Table 5), yet a positive nitroprusside test was apparent at this concentration. Ten millimolar cysteine significantly decreased germination by C. albicans as has been reported (111).

Both TC199 and cell filtrate yielded a positive nitroprusside test before anion-exchange column chromatography but were negative after passage through the column, implying that compounds containing free sulfhydral groups were either retained on the column or oxidized during passage through the column. Yet, the anion-exchange eluate of a cell filtrate maintained 68% of the inhibitory activity obtained before chromatography. TC199 did not show and inhibitory activity after anion exchange. These data indicate that the regulatory factor is not cysteine.

At concentrations of trytophol and PEA shown to be inhibitory by Lingappa at al. (1969) and at five times these concentations, inhibition of germination was only 19% with PEA and 0% with tryptophol. Cell growth during an 8 h incubation at 37°C was also not decreased in the presence of TC199 with 25 times the concentration of either tryptophol or PEA reported by Lingappa et al (83)

Table 5. Effect of cysteine on germination by C. albicans.

Cysteine concn	Nitroprusside reaction[b]	Germ tubes (%)[c]	Inhibition (%)[d]	Pseudo-germ tubes (%)	Yeasts (%)
TC199	+	77.7 (1.1)[e]		14	8.2
10^{-1} M	+	0 (0.0)	100	0	100
5×10^{-2} M	+	0 (0.0)	100	0.5 (0.7)	99.5 (0.7)
1×10^{-2} M	+	35.7 (3.5)	44.1	14.7 (1.5)	49.7 (1.5)
5×10^{-3} M	+	57.0 (4.9)	26.6	10.0 (4.2)	33.1 (0.8)
1×10^{-3} M	+[f]	70.5 (3.1)	9.3	15.2 (1.5)	14.3 (1.4)
1×10^{-4} M	–	78.0 (5.6)	0.0	15.1 (4.7)	6.9 (0.8)

[a] Cysteine concentrations were prepared fresh so that 0.1 ml of a concentration when added to 0.9 ml of TC199 gave the concentration indicated. The cysteine concentration in TC199 (0.1 μg/ml) was not considered to be significant. Tubes were incubated for 3 h at 37°C under 5% CO_2.

[b] Three drops of sodium nitroprusside were added to 1.0 ml of the various cysteine concentrations. A color change from clear to lavender was considered positive.

[c] Each experiment was run in duplicate. Percent values were obtained by counting 200 cells for each run.

[d] As compared to control value of 77.7%.

[e] Standard deviation of the mean of at least two separate experiments run in duplicate each time.

[f] When nitroprusside was added to 1 mM cysteine the solution turned faintly lavender, but upon slight agitation it returned to clear.

to inhibit growth.

Gas chromatography (GC) was also used to determine if tryptophol or PEA were present in cell filtrates. Neither substance was detected. When PEA or tryptophol were added to TC199 at one-tenth that used by Lingappa et al (83) to inhibit growth and analyzed by GC both substances yielded peaks. These data indicate that the germination inhibitory substance in cell filtrate is not tryptophol or PEA.

Effect of Cell Filtrates with Germination Regulatory Activity on Growth of *C. albicans*

Cell viability (colony-forming units) and total cell number (direct counts) were determined after incubation of cells in active cell filtrate made in TC199. At the end of a 3 h incubation period, the viable and total cell counts of *C. albicans* 9938 in TC199 or in cell filtrate increased slightly over the starting inoculum (118 and 104%, respectively). Cell increases of *C. albicans* incubated in either medium continued with time, and by 8 h the counts increased 157% in tubes containing TC199 and 151% in tubes containing cell filtrate relative to the cell concentation at 3 h.

Cell growth in GM-2 or cell filtrate prepared with

GM-1 and supplemented with GM-2 was also determined with the broth culture assay by comparing the milligram dry weight of cells after 4 h incubation with the milligram dry weight of cells after 30 minutes of incubation. A significant increase in dry weight by cells in cell filtrate over cells in GM-2 was detected (Table 6). This indicates that the germination regulator does not inhibit growth of cells.

Effect of Metals on Germination

All metals tested affected germination of C. albicans 9938 cells when the concentration was at least 10^{-3} M (Figure 8). Ferric chloride was least effective at inhibiting germination and $MnCl_2$ appeared most efective.

For subsequent experiments, which tested the ability of a metal to augment the germination inhibitory capacity of a cell filtrate, the highest concentration (within an order of magnitude) of metal which did not significantly affect germination was used. Hence, 10^{-4} M Fe^{3+} and Mg^{2+}, 10^{-5} M Ca^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Mo^{6+}, 10^{-6} M Zn^{2+}, and 10^{-7} M Mn^{2+} were utilized.

Table 6. Effect of cell filtrate on growth of cells during germination assay.

Medium	cell weight 30 min	cell weight 4 h	%increase[a]
GM-2	0.35 (0.14)[b,c]	0.98 (0.22)	180
Cell filtrate	0.42 (0.22)	2.96 (0.67)	604

[a] % increase= $\left(\frac{\text{dry wt of cells at 4 h}}{\text{dry wt of cells at 30 min}} \times 100\right) - 100$

[b] Values in parenthesis represent the standard error of the mean of 3 experiments, each run in triplicate.

[c] All values are in milligrams.

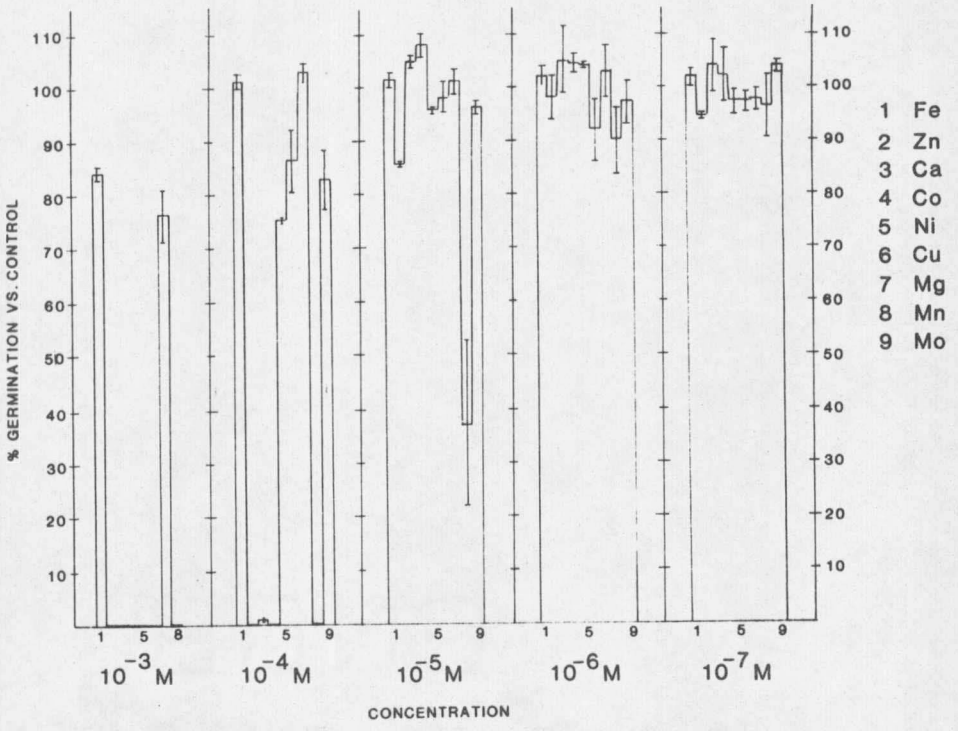


Figure 8. Effect of different concentrations of metal on germination by Candida albicans. Metal solutions were diluted into GM-2 to give the concentration indicated and germinative ability of cells was determined with the broth culture assay. Each experiment was run at least twice and triplicate cultures were tested for each concentration of metal in each experiment. Error bars indicate the standard error of the mean.

Effect of Metals on Germination Regulatory Activity

Only Co^{2+} affected the germination inhibitory activity of cell filtrate (Figure 9). The enhancement of activity appears to be due to the cobalt ion and not the counter anion because both $CoCl_2$ and $Co(NO_3)_2$ caused significant increases in germination inhibiton.

Effect of Cell Filtrate on ^{60}Co and ^{65}Zn Uptake

To determine if cobalt uptake by cells is increased in the presence of cell filtrate, ^{60}Co and $10^{-5}M$ $CoCl_2 \cdot 6H_2O$ was added to media at the beginning of an incubaiton period (t_0) and cpm/mg dry weight of cells was evaluated four hours later (t_4). No difference in uptake of ^{60}Co was obtained between cells in GM-2 and in cell filtrate when cobalt was added at t_0 (Figure 10).

When the addition of cobalt (^{60}Co and "cold" cobalt) was not made to media until the third hour of the incubation period (t_3), a significant increase in cobalt uptake by cells in cell filtrate over cells in GM-2 was seen. Furthermore, this increased uptake was significantly greater than that of cells in either cell filtrate or GM-2 given cobalt at t_0 (Figure 11).

The enhanced ^{60}Co uptake by cells in cell filtrate

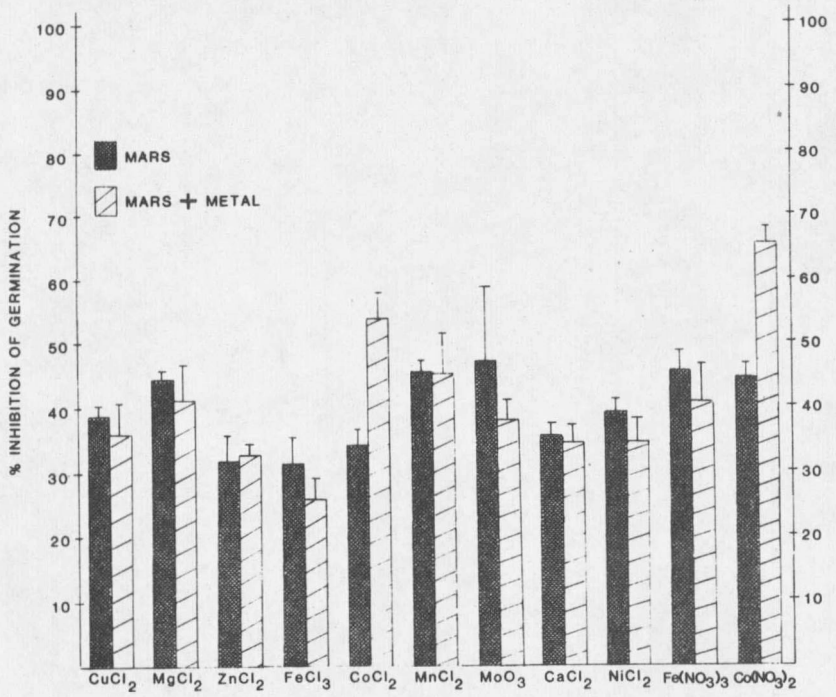


Figure 9. Effect of metals on germination inhibitory activity. Metals were added to either cell filtrate or GM-2 at the beginning of incubation in the broth culture assay. Germination was assessed after 4 h. The amount of inhibition of germination by cell filtrate with or without metal was determined by comparing the percent germination in this medium to the percent germination in GM-2 with or without metal. The results are the average of three experiments, each run in triplicate. Error bars represent the standard error of the mean.

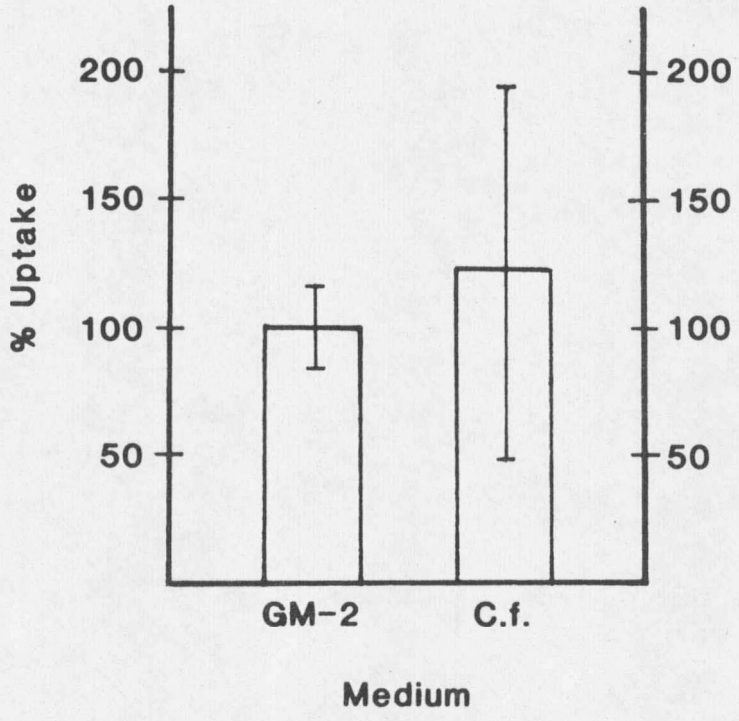


Figure 10. Effect of cell filtrate (c.f.) on uptake of ^{60}Co by cells. Two uCi of ^{60}Co plus 10^{-5} M $CoCl_2$ was added to each of triplicate cultures of cells at t_o and the cpm/mg dry weight of cells was determined at t_4. Uptake by cells in cell filtrate was compared to uptake by cells in GM-2 on a percent basis. The results are the mean of four experiments and the error bars indicate the standard error of the mean.

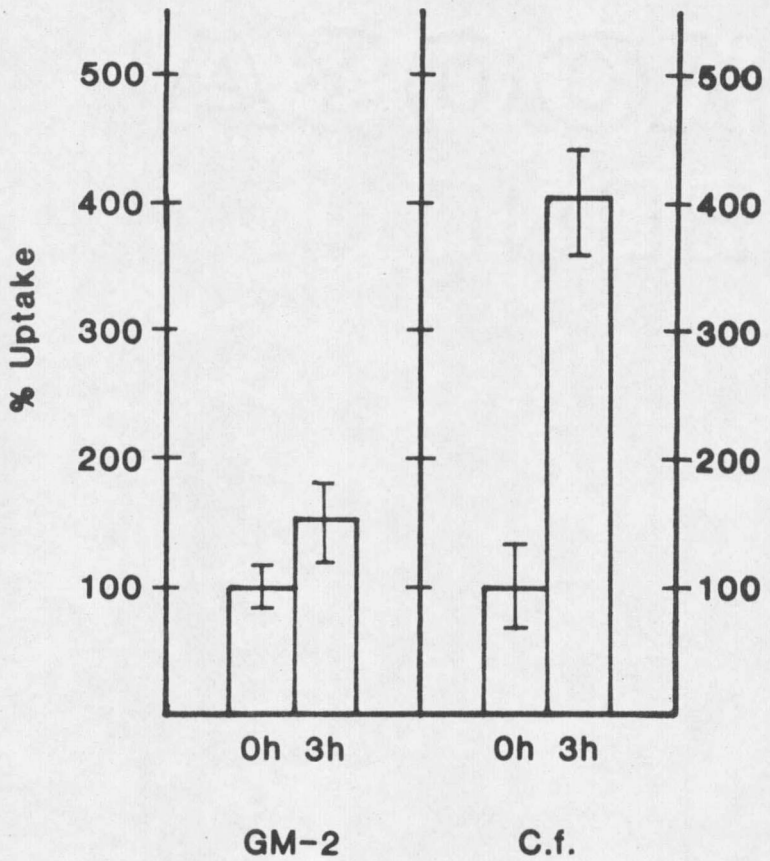


Figure 11. Effect of delayed addition of cobalt on uptake by cells in GM-2 and cell filttrate. Cobalt was added to broth cultures at either t_o or t_3 and the cpm/mg dry weight of cells was determined at t_4. The amount of ^{60}Co associated with cells given cobalt at t_3 was compared to cells in similar medium given cobalt at t_o. The results are the mean of four experiments run in triplicate. Error bars are the standard error of the mean.

was also seen if the addition of cobalt was made at different times during the 4 h incubation period (Figure 12). Uptake was similar to that observed for cells in medium given cobalt at t_3.

When cobalt was added to cell filtrate at t_0 and t_3, uptake of ^{60}Co by cells was not increased over the uptake by cells in cell filtrate given cobalt at only t_0 (data not shown).

Other strains of *C. albicans*, *viz.*, *C. albicans* 394, 550, and 804a also gave the enhanced uptake of cobalt effect when incubated in cell filtrate (Figure 13). All three of these strains germinated well (each averaged approximately 65%) in control medium. One strain of *C. albicans*, *C. albicans* 2252, which did not germinate in GM-2, also did not show enhanced uptake of cobalt after exposure to cell filtrate. After repeated subculturing, *C. albicans* 2252 did germinate in GM-2 and did show the enhancement of cobalt uptake (Figure 13). With two other yeasts, *S. cerivisiae* and *C. tropicalis*, both of which do not germinate in GM-2, increased uptake of cobalt did not occur (Figure 13).

The enhanced uptake of cobalt by cells in cell filtrate supplemented with cobalt at t_3 was similar

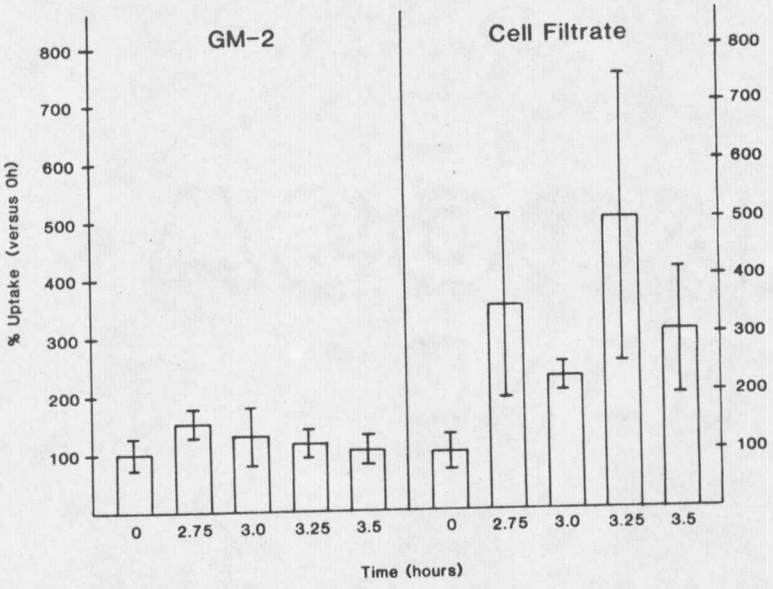


Figure 12. Effect of various delay periods on uptake of cobalt. Cobalt was added at various tiimes to either GM-2 of cell filtrate and the cpm/mg dry weight of cells was determined at t_4. The amount of ^{60}Co associated with cells given cobalt at different times was compared to that with cells given cobalt at t_o on a percent basis. Results are the mean of three experiments each run in triplicate. Error bars are the standard error of the mean.

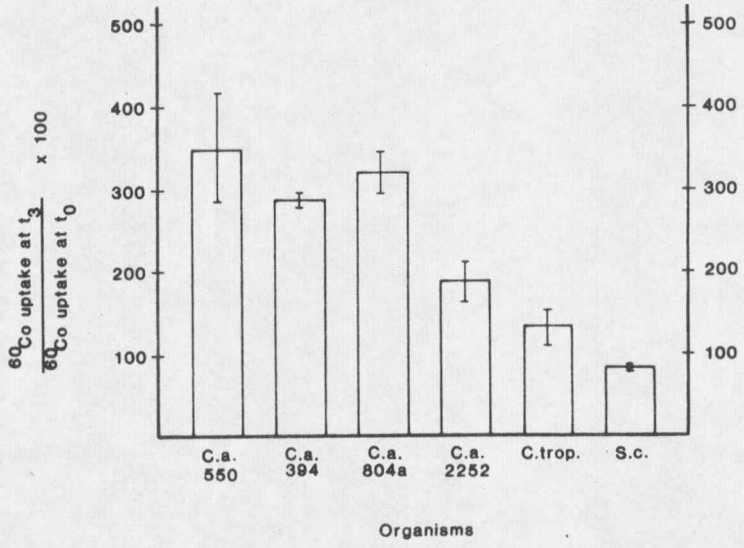


Figure 13. Test with different yeasts for enhanced cobalt uptake effect in cell filtrate. Cobalt was added to broth cultures of different yeasts at either t_o or t_3. Cpm/mg dry weight of cells was determined at t_4. The amount of ^{60}Co associated with cells given ^{60}Co at t_3 was compared with that of cells given cobalt at t_o on a percent basis. Results are the mean of two experiments, each run in triplicate. Error bars are the standard error of the mean. Organisms tested were C. albicans strains 550, 394, 804a, and 2252, Candida tropicalis (C. trop.) and Saccharomyces cerevisiae (S.c.).

between cells in acid cleaned glassware and non-acid cleaned glassware. This indicates that metals that may leach from glassware had no effect on the cobalt uptake experiments.

Incorporation of ^{60}Co into cold (0°C) 10% TCA precipitable material was found to be similar between cells in GM-2 and cell filtrate supplemented with cobalt at t_0. The amount of precipitable counts was about 50% of the total number of counts associated with non-TCA treated cells. Heat killed cells (60°C for 30 min) incorporated less than 5% of the total ^{60}Co associated with them. These results suggest that live cells contain proteins or macromolecules which bind cobalt.

^{65}Zn uptake by cells was tested in a similar fashion to cobalt. Uptake by cells in either GM-2 or cell filtrate with ^{65}Zn and 10^{-6} M $ZnCl_2$ added at t_0 or t_3 did not differ significantly (data not shown) and no difference in uptake by cells exposed to cell filtrate for 3 h prior to the addition of zinc was observed (Figure 14).

Zinc and Cobalt Levels in Media

Atomic absorption spectroscopy for zinc and cobalt

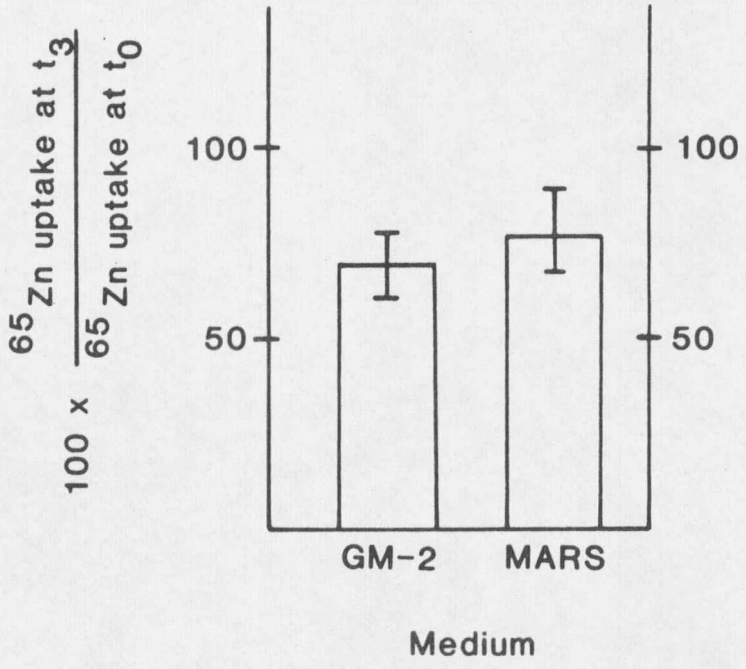


Figure 14. Effect of delayed addition of zinc to media on uptake of zinc by cells. Zinc was added to media at either t_o or t_3 and the cpm/mg dry weight of cells was determined at t_4. The amount of ^{65}Zn uptake by cells given zinc at t_3 was compared to cells in similar medium given zinc at t_o on a percent basis.

levels in GM-1 and cell filtrate revealed that zinc is present at 0.04 ug/ml (6.12×10^{-7}M) in GM-1 and 0.01 ug/ml (1.53×10^{-7}M) in cell filtrate. Cobalt concentrations were determined to be less than 0.01 ug/ml (1.7×10^{-7}M) in both media. These concentrations of zinc and cobalt did not affect germination by C. albicans in GM-2.

Effect of Cell Filtrate on Uptake of Metabolites

GM-2 and cell filtrate made with GM-1 were tested for their effect on uptake of various substances by C. albicans 394 cells in the broth culture assay. Two and four milliliter aliquots of cell suspensions were analyzed for cell associated radiolabel. Roughly similar percent uptake values for cells in cell filtrate and in control media were obtained with both the two and four ml aliquots (Tables 7, 8, 9, and 10). These results indicate that the different amounts of cells filtered onto GF/C membranes did not cause disparate quenching of particles emitted by tritium or ^{14}C atoms and, thus, cpm associated with cells in GM-2 and in cell filtrate could be compared.

The results of three glucose uptake values are shown in Table 7. In two experiments, (No. 2 and 3), glucose

Table 7. Effect of cell filtrate on uptake of ^{14}C-glucose.

Expm number	Time[a]	% germ tubes		2 ml aliquots[c]		4 ml aliquots	
		GM-2	C.F.[b]	GM-2	C.F.	GM-2	C.F.
1	30 min	0 (0)[d]	0 (0)	100[e] (25.5)	187.6 (31.2)	100 (8.3)	155.7 (34.9)
	4 h	90.0 (1.7)	6.2 (1.8)	100 (8.3)	116.6 (7.3)	100 (17.3)	119.2 (8.3)
2	30 min	0 (0)	0 (0)	100 (19.4)	69.7 (4.1)	100 (3.7)	72.5 (4.3)
	4 h	89.7 (3.2)	1.7 (0.6)	100 (12.3)	143.2 (3.4)	100 (10.2)	124.2 (15.0)
3	30 min	0 (0)	0 (0)	100 (20.1)	127.4 (19.9)	100 (27.1)	82.7 (1.5)
	4 h	92.3 (1.5)	17.0 (9.6)	100 (8.9)	68.6 (10.3)	100 (2.7)	68.2 (9.6)

[a] Time at which cells in broth culture assay were monitored.

[b] Cell filtrate

[c] Either 2 ml or 4 ml of cell suspension from each flask was assayed.

[d] Values in parenthesis are the standard deviation of triplicate cultures.

[e] Values are the percent uptake of ^{14}C-glucose relative to the cpm/100 ug dry wt of cells from GM-2 broths.

uptake at 30 minutes ($t_{30'}$) by cells in cell filtrate was not significantly different than by those in GM-2 but was slightly increased in the third experiment (No. 1). At 4 h (t_4), glucose uptake by cells in filtrate varied when compared to cells in control medium. Such variability suggests that the filtrate has no effect on the ability of cells to take up glucose.

Cells in cell filtrate relative to those in GM-2 also varied in their ability to take up ^{3}H-adenine (Table 8). Again, because the effect on cell filtrate on uptake of, in this case, adenine, was variable, it does not appear that differential adenine uptake by cells is involved in yeast formation.

However, uptake of ^{3}H-adenine was slightly less by cells exposed to cell filtrate than those in control medium (Table 9). The difference in uptake, although significant, was only about 25% at 4 h.

Cell filtrate also caused cells to take up less amino acids than cells in GM-2 (Table 10). The difference in uptake between cells in the two media was about 70%.

In the case of ^{3}H-amino acids and ^{3}H-uridine, decreased uptake by C. albicans exposed to cell filtrate (which contains a germination inhibitor) was evident by 30

Table 8. Effect of cell filtrate on uptake of ^{3}H-adenine.

Expm number	Time[a]	% germ tubes GM-2	% germ tubes C.F.[b]	2 ml aliquots[c] GM-2	2 ml aliquots C.F.	4 ml aliquots GM-2	4 ml aliquots C.F.
1	30 min	0 (0)[d]	0 (0)	100[e] (33.5)	116.2 (39.0)	100 (17.1)	185.5 (62.7)
	4 h	90.0 (1.7)	6.2 (1.8)	100 (10.6)	87.6 (8.6)	100 (44.7)	96.0 (11.1)
2	30 min	0 (0)	0 (0)	100 (13.1)	57.7 (13.4)	100 (3.5)	44.4 (7.6)
	4 h	89.7 (3.2)	1.7 (0.6)	100 (10.7)	112.4 (3.1)	100 (12.7)	111.5 (11.3)
3	30 min	0 (0)	0 (0)	100 (26.7)	115.2 (29.6)	100 (39.8)	56.9 (1.6)
	4 h	92.3 (1.5)	17.0 (9.6)	100 (7.0)	63.1 (3.0)	100 (6.6)	62.9 (9.4)

[a] Time at which cells in broth culture assay were monitored.

[b] Cell filtrate

[c] Either 2 ml or 4 ml of cell suspension from each flask was assayed.

[d] Values in parenthesis are the standard deviation of triplicate cultures.

[e] Values are the percent uptake of ^{3}H-adenine relative to the cpm/100 ug dry wt of cells from GM-2 broths.

Table 9. Effect of cell filtrate on ^{3}H-uridine uptake.

Expm number	Time[a]	% germ tubes		2 ml aliquots[c]		4 ml aliquots	
		GM-2	C.F.[b]	GM-2	C.F.	GM-2	C.F.
1	30 min	0 (0)[d]	0 (0)	100[e] (8.4)	75.5 (6.1)	100 (4.5)	62.2 (11.3)
	4 h	91.0 (3.0)	1.7 (1.2)	100 (16.4)	72.8 (3.6)	100 (20.0)	76.7 (4.6)
2	30 min	0 (0)	0 (0)	100 (11.4)	71.5 (16.6)	100 (12.4)	66.4 (10.3)
	4 h	90.3 (3.2)	2.7 (2.1)	100 (5.6)	75.8 (7.5)	100 (9.7)	94.7 (4.1)

[a] Time at which cells in broth culture assay were monitored.

[b] Cell filtrate

[c] Either 2 ml or 4 ml of cell suspension from each flask was assayed.

[d] Values in parenthesis are the standard deviation of triplicate cultures.

[e] Values are the percent uptake of ^{3}H-uridine relative to the cpm/100 ug dry wt of cells from GM-2 broths.

Table 10. Effect of cell filtrate on uptake of amino acids[a].

Expm number	Time[b]	% germ tubes GM-2	% germ tubes C.F.[c]	2 ml aliquots[d] GM-2	2 ml aliquots C.F.	4 ml aliquots GM-2	4 ml aliquots C.F.
1	30 min	0 (0)[e]	0 (0)	100[f] (12.1)	36.7 (6.0)	100 (2.6)	40.1 (12.8)
	4 h	86.7 (2.3)	2.0 (1.0)	100 (20.4)	32.9 (4.1)	100 (7.7)	33.6 (1.7)
2	30 min	0 (0)	0 (0)	ND[g]	ND	ND	ND
	4 h	87.7 (2.1)	5.3 (1.5)	100 (16.9)	26.5 (3.6)	100 (24.9)	25.8 (2.2)

[a] ^{3}H-proline, -methionine, -histidine, and -arginine

[b] Time at which cells in broth culture assay were monitored.

[c] Cell filtrate

[d] Either 2 ml or 4 ml of cell suspension from each flask was assayed.

[e] Values in parenthesis are the standard deviation of triplicate cultures.

[f] Values are the percent uptake of ^{3}H-amino acids relative to the cpm/100 ug dry wt of cells.

[g] Not done.

minutes. These results suggest that the ability of cells to take up these substances is determined within minutes after the organisms are placed into media.

Studies on incorporation of the radiochemicals were performed in preliminary experiments in which cells (C. albicans 9938) were exposed to either TC199 or cell filtrate made in TC199. In these experiments, germination and incorporation of radiochemicals by cells was evaluated after incubation under conditions described for the tube germination assay. Incorporation of ^{14}C-glucose, ^{14}C-amino acids, or ^{3}H-adenine into cold (0°C), 25% TCA precipitable material was similar by cells in both media. Amino acids were incorporated 60 to 80% less by cells exposed to filtrate.

Effect of Isolation Protocol on Activity of Germination Inhibitor

Adsorption with pretreated charcoal. The germination inhibitor in cell filtrate appeared to adsorb to charcoal because cell filtrates were no longer inhibitory to germination after charcoal treatment (Table 11).

Pyridine elution of charcoal. Pyridine elution of the germination inhibitor from charcoal was more effective with 100% pyridine than 50% pyridine in water (Table 12).

Table 11. Effect of charcoal adsorption on cell filtrate.

Fraction[a]	gt[b] (%)	ph (%)	y (%)	percent[c] inhibition
GM-2	87.8[d] (3.7)	11.8 (3.4)	1.2 (0.66)	----
lyophilized GM-1[e]	79.2 (1.8)	11.8 (4.6)	4.0 (3.0)	9.8 (2.1)
lyophilized cell filtrate	15.0 (10.7)	63.2 (16.3)	20.7 (19.0)	82.9 (12.2)
charcoal adsorbed GM-1	87.0 (3.1)	12.0 (2.2)	0.67 (0.57)	0.9 (3.5)
charcoal adsorbed cell filtrate	83.8 (5.8)	14.8 (3.8)	2.0 (1.8)	4.6 (6.6)

[a] All fractions tested at 1X concentrations of original medium with the broth culture assay.

[b] gt=germ tubes; ph=psuedohyphae; y=yeasts

[c] The inhibition of germination obtained from any fraction is based on the percent germination obtained with untreated GM-2.

[d] Results are the average of three experiments, each run in duplicate. Values in parenthesis represent the standard error of the mean.

[e] Cell filtrate was made with GM-1.

Table 12. Effect of pyridine on elution of germination autoregulatory factor from charcoal.

Fraction	medium	(%) pyridine	test concentration	gt[a] (%)	ph (%)	y (%)	percent[b] inhibition
original media	GM-2	---	1X[c]	85.9 (3.4)[d]	13.9 (3.3)	0.75 (0.53)	---
	C.f.[e]	---	1X	10.8 (8.9)	74.4 (6.4)	14.2 (8.4)	81.4 (10.3)
pyridine eluates	GM-1	50	2X	83.8 (3.2)	10.5 (4.9)	1.1 (0.75)	2.4 (3.7)
	C.f.	50	2X	73.0 (5.6)	24.5 (9.2)	3.5 (2.5)	15.0 (6.5)
	GM-1	100	2X	86.7 (3.5)	13.3 (3.5)	0 (0)	-0.9 (4.1)
	C.f.	100	2X	64.5 (7.3)	35.2 (7.8)	0.6 (0.3)	24.9 (8.5)
	GM-1	100	10X	88.2 (7.4)	10.8 (6.0)	1.4 (1.0)	-2.6 (8.6)
	C.f.	100	10X	13.1 (10.7)	73.7 (10.3)	15.4 (10.9)	84.7 (12.5)

Table 12 (cont'd).

[a] gt=germ tubes; ph=pseudohyphae; y=yeasts

[b] The inhibition of germination obtained from any fraction is based on the percent germination obtained with untreated GM-2.

[c] Fractions were dried, resolubilized with water, and diluted with GM-2 to give the concentration indicated relative to the original medium.

[d] Values in parenthesis represent the standard errors of the mean of three experiments each run in duplicate.

[e] cell filtrate

[f] pyridine was diluted 1:1 with deionized water to give 50% pyridine.

One hundred percent pyridine, however, did not appear to elute all the germination inhibitory substance adsorbed to charcoal because the eluate had to be concentrated ten fold in order to get equivalent amounts of inhibition of germination relative to the original cell filtrate.

Ether extraction of pyridine eluates. Germination inhibitory activity of pyridine eluates of GM-1 or cell filtrate was associated with only the ether phase of cell filtrate (Table 13). Furthermore, ether extraction did not cause any diminution of germination inhibitory activity of pyridine eluates. Based on these results only the ether phases of GM-1 and cell filtrate were used in subsequent purification steps.

HPLC of ether extracted material. HPLC separation was done on concentrated, ether fractions of pyridine eluates. Several UV (λ_{254}) detectable fractions were obtained from cell filtrate (Figure 15), most of which occurred within the first five minutes after sample injection. The eluate obtained after HPLC was divided into five fractions, designated A through E. Numerous runs were made and each time the five fractions were collected and pooled with their respective counterparts. Each pooled fraction was dried by flash evaporation and

Table 13. Effect of ether extraction on fractionation of pyridine eluates.

Fraction	medium	test concentration	gt[a] (%)	ph (%)	y (%)	percent[b] inhibition
original media	GM-2	1X[c]	85.9 (3.4)[d]	13.9 (3.3)	0.8 (0.5)	---
	C.f.[e]	1X	10.8 (8.9)	74.4 (6.4)	14.2 (8.4)	81.4 (10.3)
pyridine eluate	GM-1	10X	88.2 (7.4)	10.8 (6.0)	1.4 (1.0)	-2.6 (8.6)
	C.f.	10X	13.1 (10.7)	73.7 (10.3)	15.4 (10.9)	84.7 (12.5)
water phase of ether extract	GM-1	10X	85.7 (6.7)	11.5 (5.1)	1.7 (1.0)	0.2 (7.7)
	C.f.	10X	83.0 (3.4)	15.0 (3.2)	2.0 (1.3)	3.4 (4.0)
ether phase of ether extract	GM-1	10X	81.5 (4.7)	16.5 (2.1)	2.0 (1.3)	5.1 (5.5)
	C.f.	10X	12.4 (10.5)	84.3 (13.3)	7.1 (4.2)	85.5 (12.2)

Table 13 (cont'd).

[a] gt=germ tubes; ph=pseudohyphae; y=yeasts

[b] The inhibition of germination obtained from any fraction is based on the percent germinaiton obtained with untreated GM-2.

[c] Dried pyridine eluates were resolubilized with ether saturated water and extracted three times with equal volumes of diethyl ether. The ether phases were combined, dried, resolubilized with water, and diluted into GM-2 for broth culture germination assay.

[d] Values in parenthesis represent the standard error of the mean of three experiments run in duplicate.

[e] Cell filtrate.

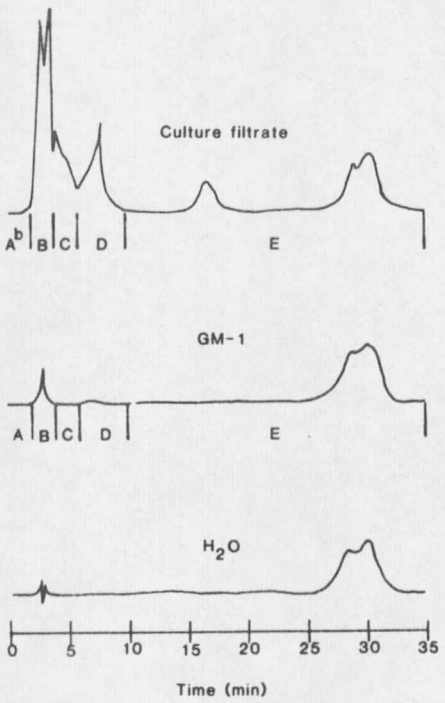


Figure 15. HPLC eluate profiles ($\lambda_{254\ nm}$) for cell filtrate (top), GM-1 (middle), and H_2O (bottom). Two hundred and fifty microliter injections of 1000X concentrated GM-1 or cell filtrate or of deionized water were made at time zero and run for 35 minutes. A step solvent gradient program was used. Solvent concentrations ranged from 50% methanol in water to 100% methanol.

lyophilization. To test each fraction for germination inhibitory activity, ESW was added to dried material and the ether was removed by bubbling N_2 through the solution. Each fraction was tested at ten or twenty-five times the concentration of the original medium. Although some inhibition was associated with fraction B of cell filtrate, only fraction C caused a significant redcution in germination (Table 14).

Control medium (GM-1), which had been fractionated in a similar manner as cell filtrate, was also chromatographed by HPLC. Few UV absorbing peaks were observed (Figure 15). The eluate was fractionated like the eluate of cell filtrate and tested for germination inhibitory activity at twenty-five times the concnetration of the original GM-2. No fraction was inhibitory to germination. Based on these results, fraction C was considered to contain the active component of cell filtrate and was thus the fraction put through subsequent purification steps.

Because control medium yielded so few peaks with HPLC and no inhibitory activity, this medium was not subjected to further isolation steps.

In order to ascertain whether any peaks present in

Table 14. Germination test of HPLC fractions of cell filtrate.[a]

Fraction	Expm 1 (10X)[b] gt[c] (%)	ph (%)	y (%)	Expm 2 (25X) gt (%)	ph (%)	y (%)
GM-2[d]	82.0	17.5	0.5	92.0	7.0	1.0
C.f.	3.5	77.5	18.0	NT[e]	NT	NT
A[f]	87.5	12.5	0	80.0	19.0	1.0
B	75.5	19.5	5.0	53.5	42.5	4.5
C	58.5	36.0	5.5	6.5	80.0	13.5
D	81.5	18.0	0.5	70.0	30.0	0
E	83.5	16.0	0.5	82.0	18.0	0

[a] Each fraction was run in duplicate cultures and each experiment represents a different preparation of material.

[b] Concentration at which each fraction tested based on the concentration in original cell filtrate.

[c] gt=germ tubes; ph=pseudohyphae; y=yeasts

[d] GM-2 and cell filtrate were tested at 1X.

[e] Not tested

[f] See "Results" for fractionation of HPLC eluate of cell filtrate.

the elution profile obtained after HPLC were due to artifact, 250 ul deionized water (the same water that is used for HPLC solvent) was run through the HPLC. The elution profile of the water after HPLC (Figure 15) indicates that some smaller peaks in fraction B and the large peak in fraction E of GM-2 and cell filtrate are artifactual.

Sephadex LH-20 chromatography of HPLC fraction C. HPLC fraction C of cell filtrate was lyophilized and then resolubilized with approximately 1.5 ml of 90% methanol in 0.05 M ammonium acetate. The resolubilized material was run through a Sephadex LH-20 column (1.5 x 31 cm) and eighty 1.0 ml fractions of the eluate were collected and their optical density at 270 nm (OD_{270}) was determined. Four UV absorbing peaks were obtained (Figure 16) and these were designated C_1, C_2, C_3, and C_4. The 80 ml eluate was divided into five portions designated C_A (fractions 1-->30), C_B (fractions 30-->45), C_C (fractions 45-->57), C_D (fractions 58-->70), and C_E (fractions 71-->80). The 1 ml fractions composing each portion were pooled, dried by flash evaporation and lyophilized, resolubilized with deionized water, and tested for germination inhibitory activity with the broth culture

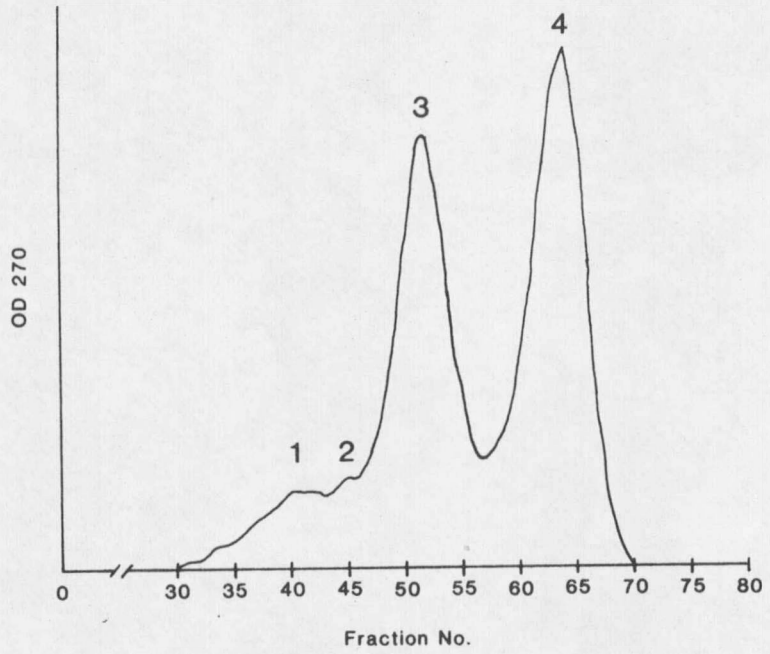


Figure 16. Elution profile (λ_{270nm}) of HPLC fraction C after LH-20 chromatography. Dried sample was resolubilized with 1.5 ml of 90% methanol in 0.05 M ammonium acetate and the solution was chromatographed through a 1.5 x 31 cm column bed of Sephadex LH-20 (Pharmacia). The mobile phase was also 90% methanol in 0.05 M ammonium acetate. The flow rate was 0.25 ml per minute and one milliliter fractions were collected. Peaks were designated as C_1, C_2, C_3, or C_4.

assay. Only C_D, which contains peak C_4, yielded inhibitory activity (Table 15). Although the results presented are for only one experiment, similar results were obtained when C_C and C_D from other LH-20 runs were tested.

Although the eluate profile presented in Figure 16 shows the presence of two peaks C_1 and C_2, reproducibility of these two peaks in other LH-20 runs was not consistent. On occasion, only one peak was obtained or, rarely, no peak.

Also, the height of peak C_4 relative to C_3 varied with many runs, C_4 was higher than C_3. When C_4 was smaller than C_3, its area (by triangulation) was never less than the area of C_3.

LH-20 eluates corresponding to peak C_4 from several runs were pooled, dried by flash evaporation and lyophilization, and rechromatographed over the LH-20 column in order to remove any contaminants (due to overlap of peaks C_3 and C_4) from material representing peak C_4. The eluate profile (Figure 17) shows that peak C_3 is no longer present. Only peak C_4 remains. The fractions representing the rechromatographed peak C_4, designated C_4-2, were pooled and dried by flash evaporation and

Table 15. Germination inhibitory activity of LH-20 fractions of cell filtrate.

Fraction[a]	gt[b] (%)	ph (%)	y (%)	percent[c] inhibition
GM-2[d]	88.5[e]	11.5	1.0	---
C_A	86.5	11.5	2.0	2.3
C_B	91.0	8.0	1.0	0
C_C[f]	90.0	9.0	1.0	0
C_D[f]	72.0	27.0	1.0	18.6
C_E	90.5	9.5	0	0

[a] Concentration tested was 10X that of the original cell filtrate.

[b] gt=germ tubes; ph=pseudohyphae; y=yeasts

[c] Based on the percent germination obtained with cells in GM-2.

[d] Concentration at which GM-2 was tested was 1X.

[e] Mean of duplicates of one experiment.

[f] Fractions C_C and C_D from other samples of HPLC fraction C were tested for germination inhibitory activity. In these experiments (3), only fraction C_D (which contains peak C_4) was active.

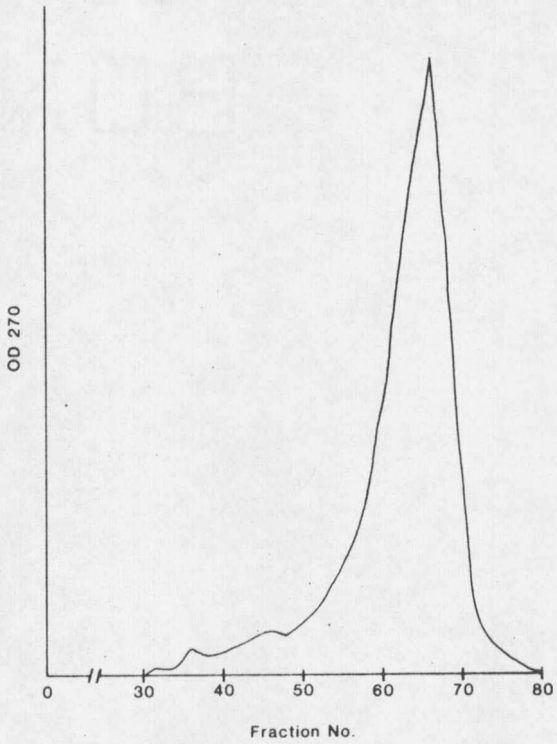


Figure 17. Elution profile (λ_{270nm}) of material corresponding to LH-20 peak C_4. Dried material was resolubilized with 1.5 ml of 90% methanol in 0.05 M ammonium acetate and the solution was chromatographed through a 1.5 x 31 cm column bed of Sephadex LH-20 (Pharmacia). The mobile phase was also 90% methanol in 0.05 M ammonium acetate. The flow rate was 0.25 ml/min., and 1 ml fractions were collected.

lyophilization.

Purification Determination of Material Representing Peak C_4-2

Material corresponding to peak C_4-2, which had been repeatedly resolubilized with water and lyophilized to remove residual ammonium acetate, was resolubilized with hot (80°C) water (approximately 0.5 mg/ml) and tested for homogeneity by TLC.

Approximately 64 ug of C_4-2 was spotted onto each of five Silica Gel IF (J.T. Baker Chem. Co.) TLC plates and developed in five different solvents. With all solvents only one spot was visible after spraying the plates with ninhydrin (Table 16). These data indicate that C_4-2 is homogeneous.

Recovery of Active Material

Table 17 indicates the amount of material harvested after a complete run through the isolation procedure (Figure 18) and its specific activity.

UV-Visible Spectroscopy of C_4-2

UV-Vis absorption maxima of C_4-2 (approximately 2 ng/ml) were determined with a Gilford Model 250 Spectrophotometer (Gilford Instrument Labortories, Inc., Oberlin, OH). Two

Table 16. TLC of C_4-2 with five different solvents.

Solvent[a]	# of spots[b]	R_f[c]	color
PDA	1	0	yellow
n-butanol	1	0	yellow with red halo
BAW	1	0.16	yellow
BMA	1	0.03	yellow
EA	1	0	yellow with red halo

[a] DPA=petroleum ether: diethylether: acetic acid (90:10:1)
BAW=n-butanol: acetic acid: water (4:1:1)
BMA=benzene: methanol: acetic acid (45:8:8)
EA=ethanol (95%): NH_3 (25%) (4:1)

[b] Visualization reagent was ninhydrin (155).

[c] $R_f = \frac{\text{distance of spot from origin}}{\text{distance of solvent front from origin}}$

Table 17. Isolation of germination inhibitor.

Purification step	total dry weight(mg)	units[a]	sp. act. (ug/unit)
4 liter cell filtrate	3508	2500	1323
LH-20 (C_4-2)	0.15	50	3[b]

[a] A unit is defined as the amount of material that inhibits germination by 18%.

[b] The activity of this fraction is increased three to five fold when combined with the methanol insoluble material obtained after lyophilization of ether extracts (see appendix 2). Hence, the specific activity may be 0.6 ug/unit.

Figure 18. Flow diagram for isolation of germination inhibitor.

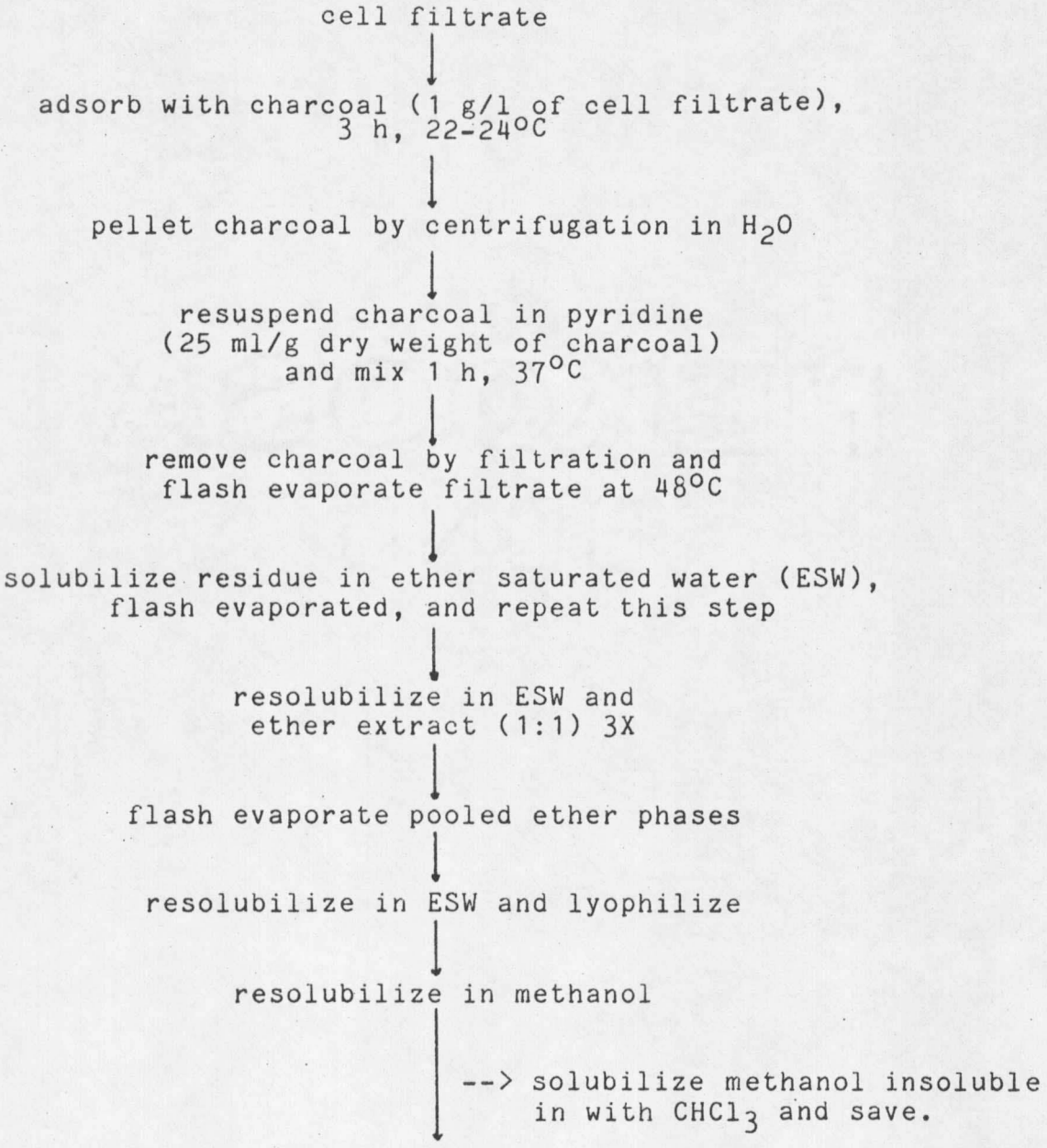
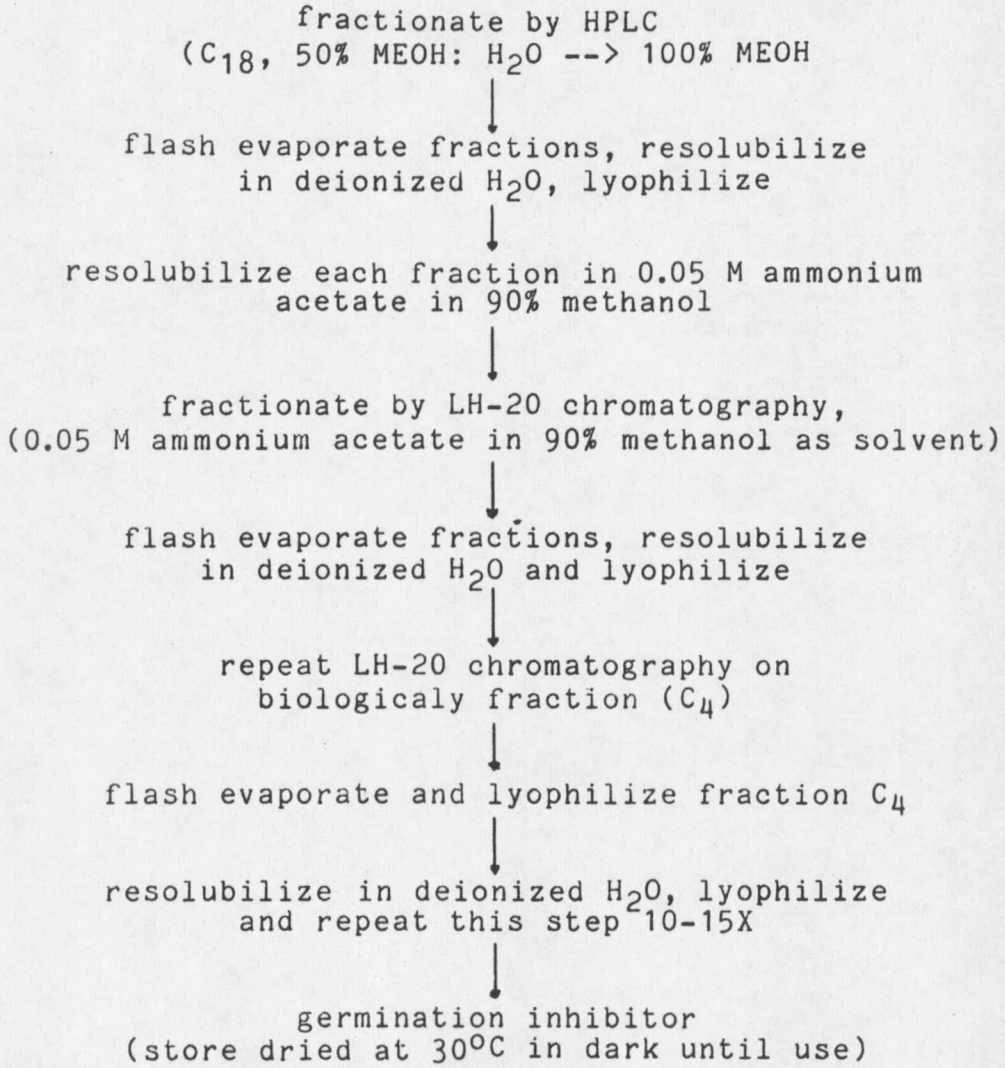


Figure 18, cont.



maxima were obtained (λ_{225} and λ_{270nm}).

PMR Spectroscopy of Various Fractions

Proton magnetic resonance spectroscopy was done on HPLC fraction C (Figure 19) as well as on LH-20 peaks C_3 (Figure 20), C_4 (Figure 21), and C_4-2 (Figure 22). To prepare C_3 and C_4-2 for PMR, dried fractions were twice resolubilized with D_2O (Sigma) and lyophilized. All dried fractions (approximately 2 mg each), which were yellow, were resolubilized with d_6-DMSO (Sigma). In the scans for all fractions, a large peak is present at δ 3.3. This is due to DHO or H_2O. Because of the size of this peak, any genuine peaks which appear in this region would be masked. Based on the scans presented in Figures 19 through 21, it appears that LH-20 chromatography did cause separation of the components in HPLC fraction C (for example, peaks at δ6.65 and δ6.95 in fraction C are not present in C_4 but are in C_3. The scan for C_4-2 (Figure 22) indicates that the germination inhibitor may contain a heterocyclic moiety. However, because of the large peaks at δ 3.3 (DHO) and δ1.6 it is difficult to interpret the spectrum.

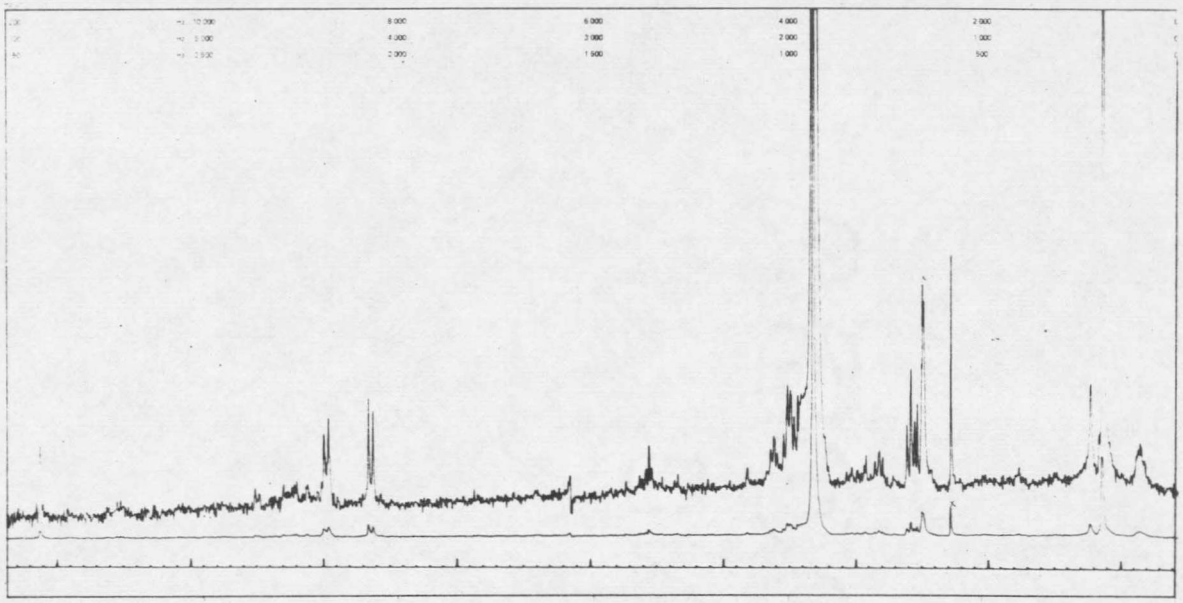


Figure 19. Proton magnetic resonance spectrum of HPLC fraction C. Dried sample was resolubilized in a small volume (< 2.0 ml) of d_6-DMSO (Sigma) and analyzed with a Bruker 250 MHz NMR. The large peak at 3.3 ppm is probably due to DHO.

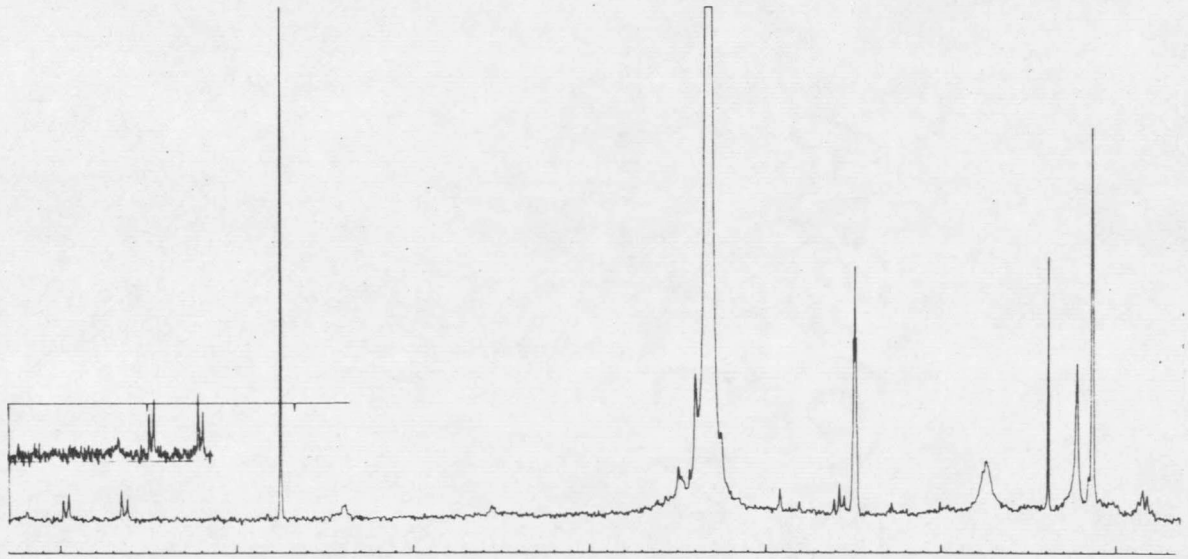


Figure 20. Proton magnetic resonance spectrum of material corresponding to peak C_3 from LH-20 chromatography of cell filtrate. Dried sample was resolubilized in a small volume (< 2.0 ml) of d_6-DMSO (Sigma) and analyzed with a Bruker 250 MHz NMR.

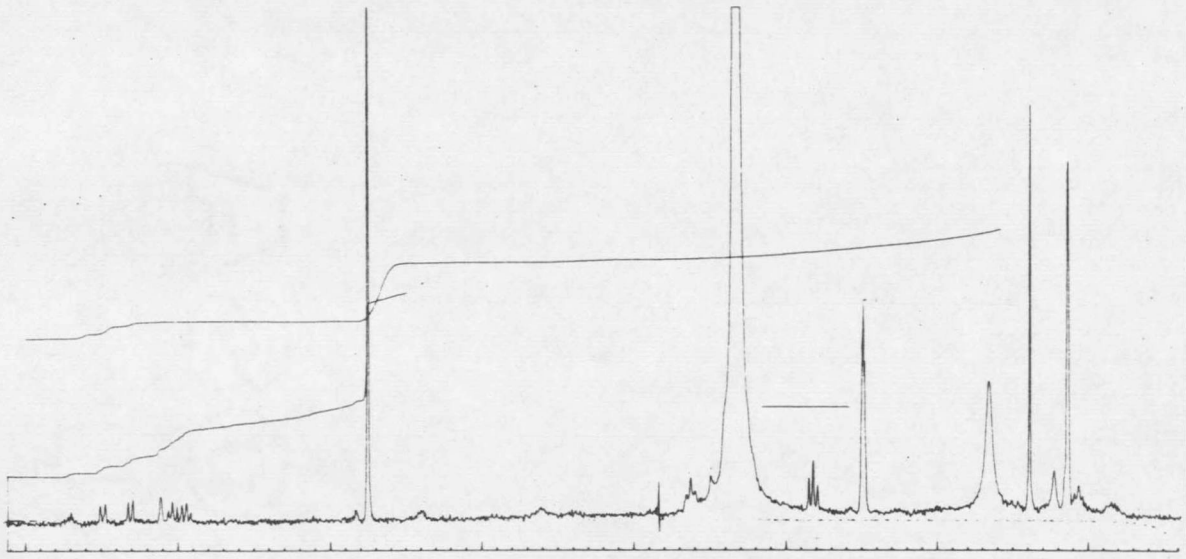


Figure 21. Proton magnetic resonance spectrum of material corresponding to peak C_4 from LH-20 chromatography of cell filtrate. Dried sample was resolubilized in a small volume of (<2.0 ml) d_6-DMSO (Sigma) and analyzed with a Bruker 250 MHz NMR.

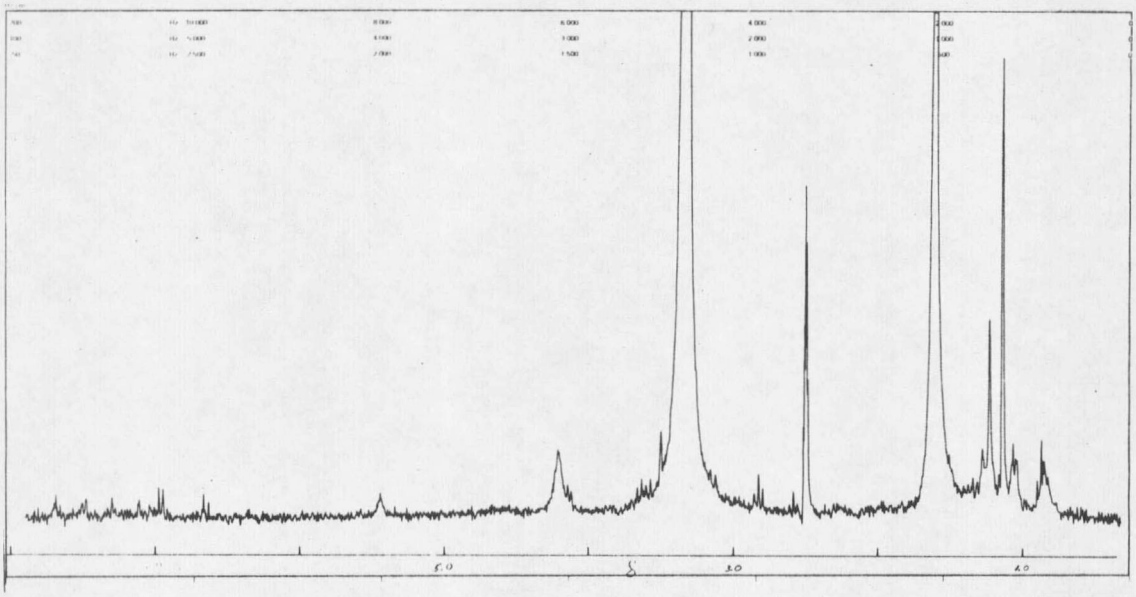


Figure 22. Proton magnetic resonance spectrum of material corresponding to peak C_4-2. The dried sample had been twice solubilized in D_2O and dried prior to solubilization in a small volume (<2.0 ml) of d_6-DMSO (Sigma). Analysis was done with a Bruker 250 MHz NMR.

DISCUSSION

Regulatory mechanisms of dimorphic change in C. albicans is a complex problem. Many investigations have been done during the past four decades to elucidate the key metabolic events which trigger Y-->M conversion. The information gleaned from these investigations, however, is difficult to interpret because no uniform terminology exists for describing various forms of C. albicans cells. In the experiments presented in this dissertation morphological phenotypes were divided into three categories: yeasts; pseudohyphae; and germ tubes. Although these terms are commonly used, they lack standard definitions. Recently, attempts to provide strict definitions for each term have been made (97, 115). Yeast is an ovoid cell and a germ tube is any filamentous outgrowth that does not have a constricted base (that point of the tube which is proximal to the mother cell) (97) and the lateral walls are parallel throughout the tube (115). The term "pseudogerm tube" advocated by Mackenzie (85) has been discarded (97). Pseudohyphae are outgrowths which arise by elongation of blastoconidia. Hence, the base is constricted and the lateral walls are not parallel throughout the tube (97, 115).

Unfortunately, absent from these definitions are situations where a group of contiguous cells contain one or more cells which are germ tubes while others are ovoid and or elongated. When these situations arise it is unclear how to categorize the group of cells. One method is to define each cell in the group. This can be difficult because septa may not be apparent and basal constriction between cells is not obvious. A second method may be to assign an order of priority to each form present within the cell group. For instance, if a group of contiguous cells contains a germ tube, then the germ tube may be given highest priority (if one is particularily interested in the production of these forms) and the cell group is designated one category, namely germ tube, eventhough there are other cellular forms in the group. Although I have used this latter system in my experiments, it has shortcomings. Because certain cellular forms are ignored, a biased estimate of morphological development is imposed. As in other systems of counting, no attempt is made to designate the time of development of a particular cellular appendage during incubation. That is, if a cell group contains two blastoconidia, a pseudohypha, and a germ tube, it is

unclear which developed first and when. Thus, an important element of cellular development in the presence of control medium or other media may be missed.

I have chosen the definitions for germ tubes, pseudohyphae, and yeasts mentioned above and in the "Materials and Methods" section because they provide a distinction between germ tubes, the morphology of highest priority, and cell groups not producing a germ tube. Furthermore, it provides a category for cellular forms which are not fully germ tubes and not yeasts. This is not unreasonable because cells affected by the germination inhibitor discussed in this thesis do not form only yeasts but, instead, form pseudohyphae and yeasts. Based on this observation and those of others (31, 115) a germ tube (as defined above) may represent the end result of a Y-->M transition and interruption of pathways leading to this form may cause formation of various types of pseudohyphae. Thus, pseudohyphae may be a transitional form between yeasts and germ tubes. If transitional states during *C. albicans* morphogenesis do exist, then studies in which investigators were actually observing yeast to pseudohyphal conversion may have more credibility than has been previously thought (115).

In an attempt to determine at which point during growth C. albicans cells are competent to form germ tubes, GYEP was inoculated with cells, incubated for 57 h and at each hour (up to 35 h) the ability of cells to form germ tubes was assessed. As early as five hours into incubation, a low percentage of cells (ca. 10%) developed germ tubes when placed into TC199 at 37° C. Germination competency increased with increasing time of incubation in GYEP. Interestingly, when cells reached stationary phase (about 10 to 12 h into incubation), they oscillated approximately every two hours in their ability to germinate until 30 h post-inoculation. At this time the cells' germination competency stabilized and yielded the highest percentage of germinative forms. This latter result agrees with the observations of Soll and Bedell (148). Several reports have indicated that only stationary phase cells are competent to form germ tubes (24, 62, 148). However, exponential phase cells have been reported to germinate if they are placed in a germination induction medium which contains N-acetyl-D-glucosamine (95). From my results, it appears that exponential phase cells are competent to form germ tubes but to a lesser degree than stationary phase cells. Stationary phase

yeast cells are thus required in order to obtain a high percentage of germinating cells during a germ tube test.

Whereas the optimum cell concentration for germination of C. albicans cell was determined to be $5x10^5$ cells/ml, in agreement with others (68, 79, 116, 143), as the cell concentration was increased the percentage of cells which germinated decreased. This effect has also been observed for Mucor indicus (7, 45) and other fungi (45, 56, 82). Furthermore, when a high concentration of C. albicans yeast cells (i.e., high enough to inhibit germination) was separated from 5×10^5 cells/ml by a semi-permeable membrane, maximum germination by the cells at the optimum concentration was prevented. These results implied that a germination inhibitory substance was produced. This was further substantiated by experiments in which filtrates obtained after 15 to 18 h culture of an inhibitory concentration of C. albicans in TC199 were found to decrease or inhibit germination of yeast cells. The inhibition of germination by a high concentration of cells or by cell filtrate was not due to nutrient depletion or change in pH because the percent germ tube positive cells did not increase when cells were incubated in cell filtrates which had been nutritionally

supplemented and adjusted to pH 7.2 to 7.4 (which is within the optimum pH range for germination [data not shown, 4]). Furthermore, when 1 x 10^8 C. albicans yeast cells per ml of TC199 were incubated under conditions of dialysis (which may remove the regulatory substance), the number of cells which germinated increased over cells not subjected to dialysis conditions.

Because regulatory activity could be recovered from culture filtrates of C. albicans 2252 and of C. tropicalis, production of the factor is not unique to C. albicans 9938. However, it is not a universal characteristic of yeasts because neither cell filtrates of both Cryptococcus laurentii and Candida parapsilosis nor a high cell concentration of either organism affected germ tube formation of C. albicans 9938.

Monitoring production of the germination inhibitor over 24 h by a high concentration of cells indicated that the highest concentration of the inhibitor was present at 9 h. After 9 h, the level decreased gradually over the next 15 h. By 24 h only about 60% of the activity seen at 9 h was still present. This suggests that after 9 h the germination inhibitor is either chemically altered, destroyed, or metabolized by cells.

Preliminary characterization of the germination ihibitory factor indicated that it is inactivated by low and high pH (4.0 and 9.5, respectively), but it retains activity after heating at 70°C or lyophilization. Although the mechanism of action of the factor is unclear, it does not appear to inhibit replication because in the presence of the factor, cells continued to divide in the yeast form. Indeed, growth in the presence of the factor (made in GM-1) was greater than in the presence of control medium (GM-2) suggesting that one action of the regulatory factor is to promote cell growth.

Autoinhibition of germination by C. albicans cells at closely apposed sides of two parallel streaks on agar-coated slides (80), the so-called "Langeron effect", has been suggested to be due to the release of substances containing free -SH groups into the growth medium (105). In accordance with these observations, Nickerson and Van Rij (111), have demonstrated that cysteine at a concentration of 10 mM inhibits germination. Germination of C. albicans 9938 was also inhibited by this concentration of cysteine, although the degree of inhibition was less than that obtained with a typical cell filatrate of C. albicans (25% vs 53%). Futhermore, only

9% inhibition occurred when 1 mM cysteine was tested and this concentration yielded only a weak sodium nitroprusside reaction (which colorimetrically detects free sulfhydral groups). The eluate obtained by passage of an inhibitory cell filtrate through an anion-exchange column was negative when tested with the nitroprusside reagent, but it still had germination inhibitory activity. These data indicate that the inhibitor is not cysteine and that the mechanism of inhibition is not due to free -SH groups.

Tryptophol and PEA have been reported to act as "autoantibiotics" of C. albicans because these substances inhibited both growth and germination (83). These substances did not affect C. albicans 9938 at the reported concentrations. Furthermore, using detection by gas chromatography it was found that neither substance is detectable in filtrates contatining the germination inhibitor. Although C. albicans strain variation may account for these descrepancies, results similar to those presented in this dissertation have been obtained by others (51).

Based on the experiments discussed so far, it is evident that C. albicans produces a factor which

suppresses germination and promotes cell growth. This factor has not been previously described for C. albicans and will henceforth be designated morphogenic autoregulatory substance or, simply, MARS.

MARS was isolated by a modification of the procedure of Sakagami et al (133). The yield of homogeneous material from four liters of original culture filtrate was approximately 0.15 mg with a specific activity of 3 ug/unit. The isolation procedure resulted in a substantial loss of activity units, some of which could be due to separation of MARS from a methanol insoluble substance(s) which is required for full activity to be expressed (see Appendix 2).

When peak C_4-2, which contains MARS, was analyzed by TLC with five different solvent systems, only one spot could be visualized with ninhydrin, indicating that C_4-2 is homogeneous. The spot was yellow. A yellow spot with ninhydrin is characteristic of proline (154). A proline standard was also chromatographed by TLC but its R_f was different from C_4-2 with all solvents (data not shown). Proline, however, stimulated germination of C. albicans, hence MARS may instead be an analogue or derivative of proline. Infrared spectroscopy (see Appendix IV) has

revealed that either an amide or a secondary amine group is present in MARS (or the contaminant), further supporting the notion that MARS contains proline-like characteristics.

As mentioned above, proline is an effective inducer of germination for C. albicans (35, 67, 76). Proline analogs, on the other hand, cause proline uptake by C. albicans cells to be inhibited and thus, also cause germination to be inhibited (32, 35). Based on the analytical data, MARS may be a derivative of proline, and may possibly be a proline analog. However, unlike other naturally produced proline analogs which inhibit growth (32), MARS may stimulate cell replication.

The mechanism of MARS effect on cells was investigated with uptake studies involving radiolabelled substances (uridine, amino acids, glucose, and adenine). Particularly notable is that cells exposed to germination inhibitor took up and incorporated less amino acids than cells in control medium. Of the four amino acids added to media, proline, histidine, and arginine enter metabolism via α-ketoglutarate. Amino acids belonging to this group are potent stimulators of germination (76). Hence, if MARS is a proline analog, it may act by preventing

transport into the fungus of proline and proline-related amino acids.

No attempt was made to determine if the amino acids taken up by cells in either control medium or medium containing MARS were incorporated into protein. Dabrowa et al (33) have reported that protein synthesis is similar between M and Y forms of C. albicans. Brummel and Soll (20) have further shown that protein synthesis rates between yeast forming and mycelial forming cells are similar but that protein synthesis is delayed during Y-->M conversion.

Adenine uptake and incorporation by MARS exposed cells was similar to cells in control medium. Similar results have been observed by others (62) suggesting that adenine metabolism is not a determinant of morphogenic change in C. albicans.

Likewise, glucose uptake and incorporation was similar between cells in control and MARS containing medium. These results do not disagree with reports that the concentration of glucose in media affects morphology of C. albicans (62, 76, 143), because I did not use different concentrations of glucose. However, based on my results, increased glucose uptake and incorporation is not

sine qua non for yeast formation.

Cells exposed to MARS took up less uridine than those in control medium. The difference, although statistically significant, was only about 25%. However, incorporation of uridine by cells in either medium was similar. It is difficult to relate these data to metabolic events occurring in Y and M forming cells because uridine may not be a specific precursor to RNA synthesis (140) and the specific products of uridine incorporation were not determined.

In a different set of experiments, I evaluated the effect of various metals to augment or suppress the germination inhibitory effect of MARS. Of nine metals tested, none decreased MARS activity. Inhibition of germination by MARS, on the other hand, was enhanced by cobalt. Other metals, which have been implicated to influence dimorphism of C. albicans, such as iron (78), zinc (9, 149, 168), and copper (157), did not affect MARS activity. This suggests that cobalt, but not other metals, is related to MARS activity. This is further supported by the observation that cells which have been incubated in medium containing MARS for 3 h prior to the addition of cobalt will take up more cobalt during the next hour than

cells exposed MARS and cobalt throughout the 4 h incubation period or cells in control medium given cobalt at either the beginning or during incubation.

The observation that no metal, except cobalt, influenced MARS activity is interesting because, as mentioned above, there are reports indicating that morphology of C. albicans can be influenced by various metals, especially zinc (9, 149, 168). However, in those experiments which demonstrated that zinc has a role in morphological transformation of C. albicans, a temperature shift of 25°C to 37°C was used to induce germination. In my experiments, which were done solely at 37°C, zinc neither changed the final morphological effect of MARS nor was taken up in different quantities by cells exposed to MARS versus cells in control medium.

The enhanced uptake of cobalt by MARS exposed cells appears to be related to germination because: 1. one strain of C. albicans (strain 2252), which did not germinate, also did not take up more cobalt after being exposed to MARS for 3 h prior to addition of cobalt (data not shown); 2. this same strain did show the enhanced cobalt uptake effect in the presence of MARS after germination ability was restored (by repeated subculturing

in GYEP (Figure 13); and 3. two other yeasts, both of which do not germinate, also did not show the enhanced cobalt uptake effect.

The relationship of cobalt and morphogenesis of C. albicans and of other dimorphic fungi has been demonstrated before. In 1949, Nickerson and Van Rij (111) presented evidence that cobalt stimulated mycelial formation by C. albicans. Reasons which could explain the disparity between their results and mine include: 1. use of different germination assays (solid medium versus broth); 2. different incubation temperatures used for inducing germination; 3. different strains of C. albicans; and 4. different criteria for categorizing cellular morphologies. (Based on drawings of cells presented by Nickerson and Van Rij (111), I believe they were studying pseudohyphal and not germ tube formation). In another study, however, cobalt was shown to cause a C. albicans mutant, which produced some mycelia at 25°C, to form "blunt ended, shortened mycelia" when the mutant was incubated in medium containing 9 or 90 uM cobalt (9). Cobalt also affects cellular morphology of other fungi. For example, Mucor indicus (formerly Mucor rouxii) will form mycelial cells under CO_2 when EDTA is present.

However, when cobalt is added along with EDTA to the medium used for growth of the organism, the effect of EDTA on cells is negated (7).

My studies show that cobalt or a cobalt binding substance may play an important role in morphogenesis of C. albicans. Some cobalt binding substances of metabolic importance are known, for instance vitamin B_{12} (138), -oxo-glutarate dehydrogenase (162), dihydrolipoic acid (161). Cobalt has been demonstrated to promote morphological changes in non-fungal systems (58, 65, 98).

The regulation of morphological conversion by C. albicans is a complex problem, the solution to which may be medically beneficial by providing alternative approaches to treatment of candidiasis. Information about morphological regulation mechanisms may also shed light on similar mechanisms in other dimorphic fungi.

My research indicates that C. albicans produces a germination regulator, namely MARS, in vitro. One advantage MARS will allow investigators is that Y and M formation can be studied under conditions, that would otherwise lead to only Y-->M conversion. Many recent investigations (ex 9, 24, 89, 90, 91, 148) of C. albicans dimorphism have involved temperature shifts (25°C -->37°C

for Y--> M, respectively). Although these experiments provide valuable information concerning C. albicans dimorphism, their relevance to pathogenesis of candidiasis (in which Y-->M conversion occurs at essentially 37°C) remains unclear (see Appendix III).

It is unknown if MARS is produced in vivo. Mackenzie (85) has speculated that the presence of either yeast or hyphae in a candidal lesion may be related to the environment within the host as well as to the nature of the host's defenses. The ability of C. albicans to regulate its morphology via MARS may further indicate that the metabolic state of the organism also plays an important role in the development of disease.

CONCLUSION

Candida albicans was determined to produce a germination autoregulatory substance. A protocol for isolating the substance was devised. The isolation protocol involved charcoal adsorption, ether extraction, and chromatography. Preliminary chemical identification of the substance indicates it contains a nitrogenous functional group. The germination inhibitory activity of the substance was enhanced by cobalt. Uptake of cobalt by cells was enhanced in the presence of the germination autoregulatory only after the cells had been exposed to the substance for three hours.

LITERATURE CITED

LITERATURE CITED

1. Albert, A. (1952). Quantitative studies of the avidity of naturally occurring substances for trace metals. 2. Amino acids having three ionizing groups. Biochem. J. 50: 691-697.

2. Al-Doory, Y. (1975). The Epidemiology of Human Mycotic Diseases. Charles C. Thomas, Publisher. Springfield, Illinois.

3. Auger, P. and Joly, J. (1975). Etude de quelques aspects de la pathogenese des infections a Candida albicans. Sabouraudia 13: 263-273.

4. Auger, P. and Joly, J. (1977). Factors influencing germ tube production in Candida albicans. Mycopathologia 61: 183-186.

5. Ballman, G.E. and Chaffin, W.L. (1979). Lipid synthesis during reinitiation of growth from stationary phase cultures of Candida albicans. Mycopathologia 67: 39-43.

6. Ballou, C. (1976). Structure and biosynthesis of the mannan component of the yeast cell envelope. Advances Microbiol Physiol. 14: 93-158.

7. Bartnicki-Garcia, S. and Nickerson, W.J. (1962). Nutrition, growth, and morphogenesis of Mucor rouxii. J. Bacteriol. 84: 841-858.

8. Bartnicki-Garcia, S. and Nickerson, W.J. (1962). Isolation, composition, and structure of cell walls of filamentous and yeast-like forms of Mucor rouxii. Biochem. Biophys. Acta. 58: 102-119.

9. Bedell, G.W. and D.R. Soll. (1979). Effects of low concentrations of zinc-resistant and -sensitive pathways for mycelium formation. Infect. Immun. 26: 348-354.

10. Bedell, G.W., A. Werth, and D.R. Soll. (1980). The regulation of nuclear migration and division during synchronous bud formation in released stationary phase cultures of the yeast Candida albicans. Expm. Cell. Res. 127: 103-113.

11. Bell, A.A. and J.A. Daly. (1962). Assay and parial pruification of self-inhibitors of germination from uredospores of the bean rest fungus. Phytopathology 52: 261-266.

12. Benham, R.W. (1931). Certain monilias parasitic to man. Their indentification by morphology and by agglutination. J. Infect. Dis. 49: 183-215.

13. Bhattacharya, A., S. Banerjee, and A. Datta. (1974). Regulation of N-acetylglucosamine kinase synthesis in yeast. Biochim. Biophys. Acta. 374: 384-391.

14. Bhattacharya, A. and A. Datta. (1977). Effect of cyclic AMP on RNA and protein synthesis in Candida albicans. Biochem. Biophys. Res. Comm. 77: 1438-1444.

15. Blakeman, J.P. (1969). Self-inhibition of germination of pycnidiospores of Mycosphaerella ligulicola in relation to the temperature of their formation. J. Gen. Microbiol. 57: 159-167.

16. Block, R.J., E.L. Durrum, and G. Zweig. 1958). Amino acids, amines, and proteins. In Manual of Paper Chromatographyu and Paper Electrophoresis, 2nd edition. Academic Press Inc., New York p. 103.

17. Boguslawski, G., G. Medoff, D. Schlessinger, and G. S. Kobayashi. (1975). Histin, an RNA polymerase inhibitor isolated from Histoplasma capsulatum. Biochem. Biophys. Res. Commun. 64: 625-631.

18. Braun, P.C. and R.A. Calderone. (1978). Chitin synthesis in Candida albicans: comparison of yeast and hyphal forms. J. Bacteriol. 135: 1472-1477.

19. Brown, L.A. and W.L. Chaffin. (1981). Differential expression of cytoplasmic proteins during yeast bud and germ tube formation in Candida albicans. Can. J. Micro. 27: 580-585.

20. Brummel, M. and D.R. Soll. (1982). The temporal regulation of protein synthesis during synchronous bud or mycelium formatio in the dimorphic yeast Candida albicans. Dev. Biol. 89: 211-224.

21. Cabib, E. and F.A. Keller. (1971). Chitin and yeast budding. Allosteric inhibition of chitin synthetase by a heat-stable protein from yest. J. Biol. Chem. 246: 167-173.

22. Caroline, L., C.L. Taschdjian, P.J. Kozinn, and A.L. Schade. (1964). Reversal of serum fungstasis by addition of iron. J. Invest. Dermatol. 42: 415-419.

23. Cassone, A., N. Simonetti, and V. Strippoli. (1973). Ultrastructural changes in the wall during germ-tube formation from blastospores of Candida albicans. J. Gen. Microbiol. 77: 417-426.

24. Chaffin, W.L. and S.J. Sogin. (1976). Germ tube formation from zonal rotor fractions of Candida albicans. J. Bacteriol. 126: 771-776.

25. Chattaway, F.W., M.R. Holmes, and A.J.E. Barlow. (1968). Cell wall composition of the mycelial and blastospore forms of Candida albicans. J. Gen. Microbiol. 51: 367-376.

26. Chattaway, F.W., R. Bishop, M.R. Holmes, F.C. Odds, and A.J.E. Barlow. (1973). Enzyme activities associated with carbohydrate synthesis and breakdown in the yeast and mycelial forms of Candida albicans. J. Gen. Microbiol. 75: 97-109.

27. Chattaway, F.E., J. O'Reilly, A.J.E. Barlow, and T. Aldersley. (1976). Induction of the mycelial form of Candida albicans by hydrolysates of peptides from seminal plasma. J. Gen. Microbiol. 96: 317-322.

28. Chattaway, F.W., P.R. Wheeler, and J. O'Reilly. (1980). Purification and properties of peptides which induce germination of blastospores of Candida albicans. J. Gen. Microbiol. 120: 431-437.

29. Chattaway, F.W., P.R. Wheeler, and J. O'Reilly. (1981). Involvement of adenosine 3':5'-cyclic monophosphate in the germination of blastospores of Candida albicans. J. Gen. Microbiol. 123: 233-240.

30. Chiew, Y.Y., M.G. Shepherd, and P.A. Sullivan, (1980). Regulation of chitin synthesis during germ-tube formation in Candida albicans. Arch. Microbiol. 125: 97-104.

31. Cole, G.T. and Y. Nozawa. (1981). Dimorphism. In Biology of Conidial Fungi. Vol. 1. pp. 97-133.

32. Dabrowa, N. and D.H. Howard. (1981). Proline uptake in Candida albicans. J. Gen. Microbiol. 127: 391-397.

33. Dabrowa, N., D.H. Howard, J.W. Landau, and Y. Schechter. (1970). Synthesis of nucleic acids and proteins in the dimorphic forms of Candida albicans. Sabouraudia 8: 163-169.

34. Dabrowa, N., J.W. Landau, and V.D. Newcomer. (1965). The antifungal activity of physiologic saline in serum. J. Invest. Derm. 45: 368-377.

35. Dabrowa, N., S.S.S. Taxer, and D.H. Howard. (1976). Germination of Candia albicans induced by proline. Infect. Immun. 13: 830-835.

36. Davies, R.R. and T.J.V. Denning. (1972). Growth and form in Candida albicans. Sabouraudia 10: 180-188.

37. Denning, T.J.V. and R.R. Dabies. (1973). Candida albicans and the chemotaxis of polymorphonuclear neutrophils. Sabouraudia 11: 220-221.

38. Diamond, R.D. and R. Krzesicki. (1978). Mechanisms of attachment of neutrophils to Candida albicans pseudohyphae in the absence of serum, and of subsequent damage to pseudohyphae by microbicidal processes of neutrophils in vitro. J. Clin. Invest. 61: 360-369.

39. Diamond, R.D., R. Krzesicki, and W. Jao. (1978). Damage to pseudohyphal forms of Candida albicans by neutrophils in the absence of serum in vitro. J. Clin. Invest. 61: 349-359.

40. Domer, J.E., J.G. Hamilton, and J.C. Harkin. (1967). Comparative study of the cell walls of the yeast-like and mycelial phases of Histoplasma capsulatum. J. Bacteriol. 94: 466-474.

41. Duran, A., B. Bowers, and E. Cabib. (1975). Chitin synthetase zymogen is attached to the yeast plasma membrane. Proc. Natl. Acad. Sci. 72: 3952-2955.

42. Duran, A., E. Cabib, and B. Bowers. (1979). Chitin synthetase distribution on the yeast plasma membrane. Science 203: 363-365.

43. Eisman, P.C., S.G. Geftic, and R.L. Mayer. (1953). Virulence in mice of colonial variants of Candida albicans. Proc. Soc. Exp. Bio. Med. 82: 263-264.

44. Elin, R.J., and S.M. Wolff. (1973). Effect of pH and iron concentration on growth of Candida albicans in human serum. J. Inf. Dis. 127: 705-708.

45. Elmer, G.W. and W.J. Nickerson. (1970). Filamentous grwoth of Mucor rouxii under nitrogen. J. Bacteriol. 101: 592-594.

46. Emmons, C.W., C.H. Binford, J.P. Utz, and K.J. Kwon-Chung. (1977). Medical Mycology, 3rd Edition. Lea and Febiger. Philadelphia.

47. Eppstein, D.H. and F.H. Tainter. (1979). Germ tube growth inhibitor from Cronartium comandrae aeciospores. Appl. Env. Microbiol. 37: 143-147.

48. Esterley, N.B. (1981). Fungal infections in children. Pediatrics in Review 3: 41-49.

49. Esterly, N.B., S.R. Brammer, and R.G. Crounse. (1967). The relatitonship of transferrin and iron to serum inhibition of Candida albicans. J. Invest. Dermatol. 49: 437-442.

50. Evans, E.G.V., F.C. Odds, K.T. Holland. (1975). Resistance of Candida albicans filamentous cycle to environmental changes. Sabouraudia 13: 231-238.

51. Evans, E.G.V., F.C. Odds, M.D. Richardson, and K.T. Holland. (1975). Optimum conditions for initiation of filamentation in Candida albicans. Can. J. Microbiol. 21: 338-342.

52. Falcone, G. and W.J. Nickerson. (1956). Cell wall mannan-protein of baker's yeast. Science 124: 272-273.

53. Falcone, G. and W.J. Nickerson. (1959). Enzymatic reactions involved in cellular division of microorganisms. Proc. 4th Internat. Congress Biochem. Vienna. 6: 65-70.

54. Fineman, B.C. (1921). A study of the thrush parasite. J. Infect. Dis. 28: 185-200.

55. Grappel, S.F. and R.A. Calderone. (1976). Effect of antibodies on the respiration and morphology of Candida albicans. Sabouraudia 14: 51-60.

56. Hardcastle, R.V. and P.J. Szaniszlo. (1974). Characterization of dimorphism of Cladosporium werneckii. J. Bacteriol. 119: 294-302.

57. Hasilik, A. (1973). Perturbation of growth and metabolism on Candida albicans by 4-bromobenzyl isothiocyanate and iodoacetate. Z. Naturforsch. 28: 21-31.

58. Haskins, K.M., R.R. Zombola, J.M. Boling, Y.C. Lee, and R.H. Himes. (1980). Tubulin assembly induced by cobalt and zinc. Biochem. Biophys. Res. Comm. 95: 1703-1709.

59. Heden, D.M. and J.D. Buck. (1980). A reemphasis-germ tubes diagnostic for Candida albicans have no constrictons. Mycopathologia 70: 95-101.

60. Hendry, A.T. (1972). Inhibition of catalase activity of Candida albicans by serum. Sabouraudia 10: 193-204.

61. Hill, D.W. and L.P. Gebhardt. (1956). Morphological transformation of Candida albicans in tissues of mice. Proc. Soc. Exp. Biol Med. 92: 640-644.

62. Hmrova, M. and L. Drobnica. (1981). Induction of mycelial type of development in Candida albicans by low glucose concentration. Mycopathologia 76: 83-96.

63. Hobot, J.A. and K. Gull. (1980). The identification of a self-inhibitor from Syncephalastrum racemosum and its effect upon sporangeospore germination. Ant. V. Leewuenloek 46: 435-441.

64. Hoeprich, P.D. Candidosis. In Infectious Diseases, 3rd Ed., P.D. Hoeprich (Ed.). Harper and Row, Hagerstown, Maryland. In print.

65. Holm-Hansen, O., G.C. Gerloff, and F. Skoog. (1954). Cobalt as an essential element for blue-green algae. Physologia Planturum 7: 665-675.

66. Jillson, O.F. and W.J. Nickerson. (1948). Mutual antagonism between pathogenic fungi. Inhibition of dimorphism in Candida albicans. Mycologia 40: 369-385.

67. Johnson, S.A.M. (1954). Candida (Monilia) albicans. A.M.A. Arch. Dermatol. Syphilis. 70: 49-60.

68. Joshi, K.R., D.A. Bremner, J.B. Gavin, P.B. Herdson, and D.N. Parr. (1973). The formation of germ tubes by Candida albicans in sheep serum and trypticase soya broth. Am. J. Clin. Path. 60: 839-842.

69. Keller, F.A. and E. Cabib. (1971). Chitin and yeast budding. Properties of chitin synthetase from Saccharomyces carlsbergensis. J. Biol. Chem. 246: 160-166.

70. Kerridge, D. (1980). The plasma membrane of Candida albicans and its role in the action of antifungal drugs. In The Eucaryotic Microbial Cell, G.W. Gooday, D. Lloyd, and A.P.J. Trinci (Eds.) Cambridge University Press, Cambridge, pp. 103-127.

71. Kessler, G. and W.J. Nickerson. (1959). Glucomannan-protein complexes from cell walls of yeasts. J. Biol. Chem. 234: 2281-2285.

72. Kiehn, T.E., F.F. Edwards, and D.A. Armstrong. (1980). The prevalence of yeasts in clinical specimens from cancer patients. Am. J. Clin. Pathol. 73: 518-521.

73. Kobayashi, G.S. and P.L. Guiliacci. (1967). Cell wall studies on Histoplasma capsulatum. Sabouraudia 5: 180-188.

74. Kot, E.J., V.L. Olson, L.J. Rolewic, and D.O. McClary. (1976). An alternate respiratory pathway in Candida albicans. Ant. v. Leeuwen. 42: 33-48.

75. Kwon-Chung, K.J. and W.B. Hill. (1970). Studies on the pink, adenine-deficient strains of Candida albicans. I. Cultural and morphological characteristics. Sabouraudia 8: 48-59.

76. Land, G.A., W.C. McDonald, R.L. Stjernholm, and L. Friedman. (1975). Factors affecting filamentation in Candida albicans: relationship of the uptake and distribution of proline to morphogenesis. Infect. Immun. 11: 1014-1023.

77. Land, G.A., W.C. McDonald, R.L. Stjernholm, and L. Friedman. (1975). Factors affecting filamentaion in Candida albicans: changes in respiratory activity in Candida albicans during filamentation. Infect. Immun. 12: 119-127.

78. Landau, J.W., N. Dabrowa, V.D. Newcomer, and J.R. Rowe. (1964). The relationship of serum transferrin and iron to the rapid formation of germ tubes by Candida albicans. J. Invest. Dermatol. 43: 473-482.

79. Landau, J.W., N. Dabrowa, V.D. Newcomer. (1965). The rapid formation in serum of filaments by Candida albicans. J. Invest. Dermatol. 44: 171-179.

80. Langeron, M. and P. Guerra. (1939). Orientation de la filamentisation des champignons livuriformes cultives sur lames gelosees. Ann. Parasitol. 17: 580-589.

81. Lee, K.L., H.R. Buckley, and C.C. Campbell. (1975). An amino acid liquid synthetic medium for mycelial and yeast forms of Candida albicans. Sabouraudia 13: 148-153.

82. Lingappa, B.T. and Y. Lingappa. (1966). The nature of self-inhibition of germination of conidia of Glomerella cingulata. J. Gen. Microbiol. 43: 91-100.

83. Lingappa, B.T., M. Prasad, Y. Lingappa, D.F. Hunt, and K. Biemann. (1969). Phenylethyl alcohol and tryptophol: autoantibiotics produced by the fungus Candida albicans. Science 163: 192-194.

84. Louria,D.B. and R.G. Brayton. (1964). Behavior of Candida cells within leukocytes. Proc. Soc. Exp. Biol. Med. 115: 93-98.

85. Mackenzie, D.W.R. (1964). Morphogenesis of Candida albicans in vivo. Sabouraudia 3: 225-232.

86. Mackinnon, J.E. (1940). Dissociation in Cnadida albicans. J. Infect. Dis. 66: 59-77.

87. Macko, V., R.C. Staples, H. Gershon, and J.A.A. Renwick. (1970). Self-inhibitor of the bean rust uredospores: methyl 3,4-dimethoxycinnamate. Science 170: 539-540.

88. Magni, G. (1948). Biological significance of the pseudomycelium in asporogenous yeasts. I. Reciprocal inhibition of parallel colonies. Mycopathol. 4: 207-212.

89. Manning, M. and T.G. Mitchel. (1980). Strain variation and morphogenesis of yeast- and mycelial-phase Candida albicans in low-sulfate, synthetic medium. J. Bbacteriol. 142: 714-719.

90. Manning, M. and T.G. Mitchell. (1980). Analysis of cytoplasmic antigens of the yeast and mycelial phases of Candida albicans by two-dimensional electrophoresis. Infect. Immun. 30: 484-495.

91. Manning, M. and T.G. Mitchell. (1980). Morphogenesis of Candida albicans and cytoplasmic proteins associated with differences in morphology, strain, or temperature. J. Bacteriol. 144: 258-273.

92. Mardon, D., E. Balish, and A.W. Phillips. (1969). Control of dimorphism in a biochemical variant of Candida albicans. J. Bacteriol. 100: 701-707.

93. Mardon, D.N., S.K. Hurst, and E. Balish. (1971). Germ-tube production by Candida albicans in minimal liquid culture media. Can. J. Microbiol. 17: 851-856.

94. Marriott, M.S. (1975). Isolation and chemical characterization of plasma membranes from the yeast and mycelial forms of Candida albicans. J. Gen. Microbiol. 86: 115-132.

95. Mattia, E. and A. Cassone. (1979). Inducibility of germ-tube formation in Candida albicans at diferent phases of yeast growth. J. Gen. Microbiol. 113: 439-442.

96. McClary, D.O. (1952). Factors affecting the morphology of Candida albicans. Ann. Missouri Bot. Garden 39: 137-165.

97. McGinnis, M.R. (1980). Laboratory Handbook of Medical Mycology. Academic Press, Inc., New York.

98. Miller, C.O. (1951). Promoting effect of cobaltous and nickelous ions on expansion of etiolated bean leaf disks. Arch. Biochem. Biophys. 32: 216-218.

99. Mitchell, T.G. (1980). Opportumistic Mycoses. In Zinsser Microbiology, 7th Edition. W.K. Joklik, H.P. Willett, and D.B. Amos (Eds). Appleton-Century-Crofts, New York.

100. Mooney, D.T. and P.S. Sypherd. (1976). Volatile factor involved in the dimorphism of Mucor racemosus. J. Bacteriol. 126: 1266-1270.

101. Nickerson, W.J. (1948). Enzymatic control of cell division in micro-organisms. Nature 162: 241-245.

102. Nickerson, W.J. (1951). Physiological basis of morphogenesis in animal disease fungi. Trans. N.Y. Acad. Sci. 13: 140-145.

103. Nickerson, W.J. (1953). Reduction of inorganic substances by yeasts. I. Extrcellular reduction of sulfate by species of Candida. J. Inf. Dis. 93: 43-56.

104. Nickerson, W.J. (1954). Experimental control of morphogenesis in micro-organisms. Ann. N.Y. Acad. Sci. 60: 50-57.

105. Nickerson, W.J. and C.W. Chung. (1954). Genetic block in the cellular division mechanism of a morphoogical mutant of a yeast. Am. J. Bot. 41: 114-120.

106. Nickerson, W.J. and G. Falcone. (1956). Enzymatic reduction of disulfide bonds in cell wall protein of baker's yeast. Science 124: 318-319.

107. Nickerson, W.J. and G. Falcone. (1956). Identification of protein disulfide reductase as a cellular division enzyme in yeasts. Science 124: 722-723.

108. Nickerson, W.J. and O.F. Jillson. (1948). Interaction btween pathogenic fungi in culture. Considerations on the mechanism of cell division in the dimorphism of pathogenic fungi. Mycopath. Mycol. Applic. 4: 279-283.

109. Nickerson, W.J. and Z. Mankowski. (1953). Role of nutrition in the maintenance of the yeast-shape in Candida. Am. J. Bot. 40: 584-592.

110. Nickerson, W.J. and A.H. Romano. (1952). Enzymatic reduction of cystine by coenzyme 1 (DPNH). Science 115: 676-678.

111. Nickerson, W.J. and N.J.W. Van Rij. (1949). The effect of sulfhydral compounds, penicillin, and cobalt on the cell division mechanism of yeasts. Biochim. Biophys. Acta 3: 461-475.

112. Nickerson, W.J. and K. Zerahn. (1949). Accumulation of radiaactive cobalt by dividing yeast cells. Biochim. Biophys. Acta 4: 476-483.

113. Niimi, M., K. Niimi, J. Tokunaga, and H. Nakayama. (1980). Changes in cyclic nucleotide levels and dimorphic transition in Candida albicans. J. Bacteriol. 142: 1010-1014.

114. Nishioka, Y. and M. Silva-Hutner. (1974). Dimorphism, sensitivity to nystatin and acriflavin uptake in a strain of Candida albicans grown with glutamate as sole nitrogen and carbon source. Sabouraudia 12: 295-301.

115. Odds, F.C. (1979). Candida and Candidosis. University Park Press, Baltimore.

116. Ogletree, F.F., A.T. Abdelal, and D.G. Ahearn. (1978). Germ tube formation by atypical strains of Candida albicans. Ant. van Leeuw. J. Microbiol. Serol. 44: 15-24.

117. Olaiya, A.G., J.R. Steed, and S.J. Sogin. (1980). deoxyribonucleic acid-deficient strains of Candida albicans. J. Bacteriol. 141: 1284-1228.

118. Parker, J.C., J.J. McCloskey, and R.S. Lee. (1978). The emergence of candidosis. The dominant postmortem cerebral mycosis. Am. J. Clin. Pathol. 70: 31-36.

119. Paznokas, J.L. and P.S. Sypherd. (1975). Respiratory capacity, cyclic adenosine 3',5'-monophosphate, and morphogenesis of Mucor racemosus. J. Bacteriol. 124: 134-139.

120. Phaff, H.J. (1971). Structure and biosynthesis of the yeast cell envelope. In The Yeasts, vol. 2 A.H. Rose and J.S. Harrison (Eds.), Academic Press, Inc., New York, pp. 135-210.

121. Polak, A. and W.H. Wain. (1979). The effect of 5-fluorocytosine on the blastospores of hyphae of Candida albicans. J. Med. Microbiol. 12: 83-97.

122. Poulter, R., K. Jeffery, M.J. Hubbard, M.G. Shepherd, and P.A. Sullivan. (1981). Parasexual genetic analysis of Candida albicans by spheroplast fusion. J. Bacteriol. 146: 833-840.

123. Pugh, D. and R.A. Cawson. (1978). The effect of nystatin on the morphology of Candida albicans. Sabouraudia 16: 307-311.

124. Pugh, D. and R.A. Cawson. (1979). The induction of germ tubes in Candida albicans by an intrinsic factor. Microbios 24: 73-79.

125. Purohit, B.C., K.R. Joshi, I.N. Ramdeo, and T.P. Rharadwaj. (1977). The formation of germ tubes by Candida albicans when grown with Staphylococcus pyogene, Escherichia coli, Klebsiella pneumoniae, Lactobacillus acidophilus, and Proteus vulgaris. Mycopathologia 62: 187-189.

126. Rajasingham, K.C. and R.A. Cawson. (1980). Cell wall and plasma membrane ultrastructure of the invasive hyphae of Candida albicans. Pathology 8: 430.

127. Rao, G.R., T. Ramakrishnan, and M. Sirsi. (1960). Enzymes in Candida albicans. 1. Pathways of glucose dissimilation. J. Bacteriol. 80: 654-658.

128. Rao, G.R., M. Sirsi, and T. Ramakrishnan. (1962). Enzymes in Candida albicans. 2. Tricarboxylic acid cycle and related enzymes. J. Bacteriol. 84: 778-783.

129. Rebora, A., R.R. Marples, and A.M.Kligman. (1973). Experimental imfection with Candida albicans. Arch. Dermatol. 108: 69-73.

130. Richenberg, H.V. (1974). Cyclic AMP in prokaryotes. Ann. Rev. Microbiol. 28: 353-369.

131. Rippon, J.W. (1980). Dimorphism in pathogenic fungi. CRC Crit. Rev. Microbiol. 8: 49-97.

132. Romano, A. (1966). Dimorphism. In The Fungi, vol. 2. G.C. Ainsworth and A.S. Sussman (Eds.), Academic Press, Inc., New York, pp. 181-209.

133. Sakagami, Y., M. Yoshida, A. Isogai, and Aa. Suzuki. (1981). Peptidal sex hormones inducing conjugation tube formation in compatible mating-type cells of Tremella mesenterica. Science 212: 1525-1527.

134. Saltarelli, C.G. (1973). Growth stimulation and inhibition of Candida albicans by metabolic by-products. Mycopathol. Mycol. Appl. 51: 53-63.

135. Sarachek, A., D.D. Rhoads, and R.H. Schwarzhoff. (1981). Hybridization of Candida albicans through fusion of protoplasts. Arch. Microbiol. 129: 1-8.

136. Scharr, H., I. Long, and A. Widra. (1974). A combination rapid and standard method for identification of Candida albicans. Mycopathol. Mycol. Appl. 52: 203-207.

137. Scherwitz, C., R. Martin, and H. Ueberberg. (1978). Ultrastructural investigations of the formation of Candida albicans germ tubes and septa. Sabouraudia 16: 115-124.

138. Schroeder, H.A. (1960). Possible relationships between trace metals and chronic diseases. In Metal-binding in Medicine. M.J. Gaven and L.A. Johnson (ed) J.B. Lippincott Co., Philadelphia.

139. Schroeder, W.A. (1967). Separation of peptides by chromatography on columns of Dowex 1 with volatile developers. Methods Enzymol. 11: 361-369.

140. Shearer, G., J.C. Hubbard, and H.W. Larsh. (1980). Non-specific incorporation of nucleic acid precursors in Blastomyces dermatitidis and Histoplasms capsulaatum. Mycopathologia 72: 111-119.

141. Shepherd, M.G., Y.Y. Chiew, S.P. Ram, and P.A. Sullivan. (1980). Germ tube induction in Candida albicans. Can. J. Microbiol. 26: 21-26.

142. Shepherd, M.G., H.M. Ghazali, and P.A. Sullivan. (1980). N-acetyl-D-glucosamine kinase and germ-tube formation in Candida albicans. Expm. Mycology 4: 147-159.

143. Shepherd, M.G. and P.A. Sullivan. (1976). The produuction and growth characteristics of yeast and mycelial forms of Candida albicans in continuous culture. J. Gen. Microbiol. 93: 361-370.

144. Simonetti, N., V. Strippoli, and A. Cassone. (1974). Yeast mycelial conversion induced by N-acetyl-D-glucosamine in Candida albicans. Nature 250: 344-346.

145. Singh, B.R. and A. Datta. (1978). Glucose repression of the iducible catabolic pathway for N-acetylglucosamine in yeast. Biochem. Biophys. Res. Comm. 84: 58-64.

146. Skinner, C.E. (1947). The yeast-like fungi: Candida and Brettanomyces. Bacteriol Rev. 11: 227-274.

147. Smith, J.K. and C.B. Louris. (1972). Anti-Candida factors in serum and their inhibitors. II. Identification of a Candida-clumping factor and the influence of the immune response on the morphology of Candida and on anti-Candida activity of serum in rabbits. J. Infect. Dis. 125: 115-122.

148. Soll, D.R. and G.W. Bedell. (1978). Bud formation and the inducibility of pseudo-mycelium outgrowth during release from stationary phase in Candida albicans. J. Gen. Microbiol. 108: 173-180.

149. Soll, D.R., G.W. Bedell, and M. Brummel. (1981). Zinc and the regulation of growth and phenotype in the infectious yeast Candida albicans. Infect. Immun. 32: 1139-1147.

150. Soll, D.R., G. Bedell, J. Thiel, and M. Brummel. (1981). The dependency of nuclear division on volume in the dimophic yeast Candida albicans. Expm. Cell. Res. 133: 55-62.

151. Soll, D.R., M. Stasi, and G. Bedell. (1978). The regulation of nuclear migration and division during pseudo-mycelium outgrowth in the dimorphic yeast Candida albicans. Expm. Cell. Res. 116: 207-215.

152. Sundaram, S., P.A. Sullivan, and M.G. Shepherd. (1981). Changes in lipid composition during starvation and germ-tube formation in Candida albicans. Expm. Mycology 5: 140-147.

153. Taschdjian, C.L., J.J. Burchall, and P.J. Kozinn. (1960). Rapid identification of Candida albicans by filamentation on serum and serum substitute. A.M.A. J. Dis. Children 99: 212-215.

154. Taschdjian, C.L., F. Reiss, and P.J. Kozinn. (1960). Experimental vaginal candidiasis in mice; its implications for superficial candidiasis in humans. J. Invest. Dermatol. 34: 89-94.

155. Touchstone, J.C. and M.F. Dobbins. (1978). Practice of thin layer chromatography. John Wiley and Sons, Inc., New York.

156. Trinci, A.P.J. and C. Whittaker. (1968). Self-inhibition of spore germination in Aspergillus nidulans. Trans. Br. Mycol. Soc. 51: 594-596.

157. Vaughn, V.J. and E.D. Weinberg. (1978). Candida albicans dimorphism and virulence: Role of copper. Mycopathologia. 64: 39-42.

158. Wain, W.H., A.R. Brayton, and R.A. Cawson. (1976). Variations in the reponse to N-acetyl-D-glucosamine by isolates of Candida albicans.. Mycopathologia. 58: 27-29.

159. Wain, W.H., M.F. Price, and R.A. Cawson. (1975). A re-evaluation of the effect of cysteine on Candida albicans. Sabouraudia 13: 74-82.

160. Wain, W.H., M.F. Price, A.R. Brayton, and R.A. Cawson. (1976). Macromolecular synthesis during the cell cycles of yeast and hyphal phases of Candida albicans. J. Gen. Microbiol. 97: 211-217.

161. Webb, M. (1962). The biological action of cobalt and other metals. III. Chelation of cations by dihydrolipoic acid. Biochim. Biophys. Acta. 65: 47-65.

162. Webb, M. (1964). The biological action of cobalt and other metals. IV. Inhibition of -oxo-glutarate dehydrogenase. Biochim. Biophys. Acta. 89: 431-444.

163. Weld, J.T. (1952). Candida albicans. Rapid identification in pure cultures with carbon dioxide on modified eosin-methylene blue medium. A.M.A. Arch. Dermatol. Syphilol. 66: 691-694.

164. Wickerham, L.J. and L.F. Rettger. (1939). A taxonomic study of M. albicans with special emphasis on morphology and morhological variation (continued). J. Trop. Med. Hyg. 42: 204-216.

165. Winsten, S. and T.J. Murray. (1956). Virulence enhancement of a filamentous strain of Candida albicans after growth on media containing cysteine. J. Bacteriol. 71: 738.

166. Wolter, J.R. (1962). Endogenous fungus endophthalmitis. Arch. Opl thalmol. 68: 73-76.

167. Yamaguchi, H. (1974). Mycelial development and chemical alteration of Candida albicans from biotin insufficiency. Sabouraudia 12: 320-328.

168. Yamaguchi, H. (1975). Control of dimorphism in Candida albicans by zinc: effect on cell morpholgy and composition. J. Gen. Appl. Microbiol. 86: 370-372.

169. Youmans, G.P., P.Y. Paterson, and H. M. Sommers. (1980). The Biologic and Clinical Basis of Infectious Diseases, 2nd Edition. W.B. LSaunders Co., Philadelphia.

170. Young, G. (1958). The process of invasion and the persistaence of Candida albicans injected intraperitoneally into mice. J. Infect. Dis. 102: 114-120.

APPENDICES

Appendix I

Experiments to Determine if GYEP Contains MARS.

It is possible that C. albicans grown in GYEP take up MARS from GYEP and then release it when placed into fresh GM-1. This was tested in the following manner. The three components of GYEP, glucose, yeast extract, and peptone, were added to separate broths of GM-2 at final concentrations equal to their concentration in GYEP broth and tested for germination inhibitory with against C. albicans 394.

Both yeast extract and peptone inhibited germination (Table 18). The inhibition of germination by peptone was only 25%, yet a cell filtrate containing MARS inhibits germination 80%. Hence, peptone was not considered to contain MARS. Inhibition by yeast extract was greater (73.7%). In order to determine if the germination inhibitor in yeast extract fractionates similarly to MARS during the isolation procedure, yeast extract broth (1% in deionized water) was ether extracted (1:1) three times and the water and ether phases were tested for germination inhibitory activity with the broth culture assay. The phases were first lyophilized and resolubilized with ESW

Table 18. Effect of the components of GYEP on inhibition of germination.

Fraction added[a] to GM-2	gt[b] (%)	ph (%)	y (%)	percent[c] inhibition
None	87.5	11.5	1.0	0
glucose	91.5	7.5	1.0	0
YE[d,e]	26.5	38.5	35.0	69.7
peptone	66.0	20.0	14.0	24.6
YE-H_2O ph[c,d]	23.0	63.0	14.0	73.7
YE-ether ph[c,d]	82.0	14.0	4.0	6.3

[a] Glucose, peptone, and yeast extract stock solutions were prepared at 10X their concentration in GYEP. They were diluted 1:10 during preparation of GM-2 for testing by the broth culture assay.

[b] gt=germ tubes; ph=pseudohyphae; y=yeasts

[c] Based on the amount of germination obtained with *C. albicans* 394 cells in GM-1. The broth culture germination assay was used.

[d] YE=yeast extract, YE-H_2O ph=yeast extract, water phase from ether extraction, YE-ether ph=yeast extract, ether phase from ether extraction.

[e] Similar results were obtained with these fractions in another experiment.

(ether was removed by bubbling N_2 through solution) before being diluted into GM-2 for germination test. Only the water phase was inhibitory (Table 18) which is opposite from MARS (MARS partitions into the ether phase). This indicates that yeast extract does not contain MARS. Hence MARS is not a product taken up from GYEP by cells and then released by cells when they are placed in fresh medium.

Appendix II

Effect of Ether Extracted, Methanol Insoluble Material on Activity of MARS

Addition of methanol to the dried, ether extracted material of cell filtrate failed to resolubilize all of the material. The residue was tested for germination inhibitory activity as well as for its effect on MARS by the broth culture assay. The material was resolubilized in chloroform and the chloroform solution was mixed with water (or water containing material from HPLC fraction C) at a concentration which, when combined with pH 7.4 Tris buffer and 10X GM-2, would be approximately five times greater than cell filtrate. The chloroform was removed by bubbling N_2 through the solution. The methanol insoluble fraction inhibited germination slighty but enhanced the inhibitory activity of HPLC fraction C material several fold (Table 19). The results shown are for one experiment. In another experiment, the enhancement of MARS activity by the methanol insoluble fraction was 2.5 fold (18% versus 43%). In this experiment the fraction combined with the methanol insoluble material was from peak C_4 after LH-20 chromatography. The results indicate that MARS is more effective as a germination inhibitor when it

Table 19. Effect of combining methanol insoluble material with HPLC fraction C of cell filtrate.

Fraction[a]	gt[b] (%)	ph (%)	y (%)	percent[c] inhibition
GM-2	94.0[d]	6.0	0	----
HPLC-C	58.5	37.5	4.0	37.8
MeOH insol[e]	86.0	14.0	0	8.5
MeOH insol + HPLC-C	22.0	74.0	4.0	76.6

[a] Each fraction, except GM-2, was tested at a concentration of five times the original cell filtrate.

[b] gt=germ tubes; ph=pseudohyphae; y=yeasts

[c] The percent inhibition of germination was based on the percent germination obtained with C. albicans 394 cells in GM-2.

[d] Average of one experiment tested with duplicate cultures in the broth culture germination assay.

[e] The methanol insoluble material obtained after ether extraction and lyophilization of the ether phase was resolubilized with 2.0 ml of chloroform, added to water, and the chloroform was removed by bubbling nitrogen throught the solution. The solution was then diluted into GM-2 (with or without material from HPLC fraction C) and tested.

is combined with the methanol insoluble material obtained after ether extraction.

Appendix III

Effect of Growth Temperature on MARS Activity

Several investigators have used a temmperature shift of 25°C or 28°C to 37°C to induce germination of Candida albicans (2, 4, 8, 9). In my experiments, cells were grown at 37°C prior to germination induction at 37°C. To determine if growth may affect cellular sensitivity to MARS, Candida albicans 9938 was grown at either 25°C or 37°C in GYEP for 24 h and tested for germination ability at 37°C in the presence or absence of MARS. Cells grown at 25°C were not effected by MARS while cells grown at 37°C were (Table 20). The results also indicate that cells grown at 25°C germinated more than cells at 37°C. In another experiment, cell filtrate obtained after incubation of cells which were grown at 25°C, in GM-1 at 25°C for 15 h was as effective as cell filtrate obtained with cells grown at 37°C and incubated in GM-1 at 37°C for 15 h at inhibiting germ tubes (data not shown). These results indicate that growth temperature affects germinative ability of cells and their sensitivity to MARS. These results further suggest that cells grown at 25°C are not physiologically equivalent to cells grown at 37°C.

Table 20. Effect of growth temperature on sensitivity to cell filtrate[a].

Medium	growth[b] temp(°C)	gt[c] (%)	ph (%)	y (%)	percent[d] inhibition
GM-2	37	44.0[e]	55.0	1.0	----
C.f.[f]	37	7.0	82.0	11.0	84.1
GM-2	25	71.0	28.0	1.0	----
C.f.	25	72.0	36.0	2.0	0

[a] The broth culture germination assay was used.

[b] Temperature at which C. albicans 9938 cells were grown prior to inoculation into GM-1 for cell filtrate production at 37°C.

[c] gt=germ tubes; ph=pseudohypae; y=yeasts

[d] The percent inhibition of germinaiton is based on the percent germination obtained with cell in GM-2 grown at the same temperature.

[e] Results are the mean of one experiment run with triplicate cultures. The standard deviation in all cases never exceeded 4.0%.

[f] C.f.=cell filtrate

Appendix IV

Infrared Spectroscopy of MARS

Dried material corresponding to LH-20 peak C_4-2 (MARS) was prepared as a micro-KBr pellet and analyzed with a Digia Laboratory FT 15B-IR Spectrometer. The results, shown in Figure 23, indicate that MARS may contain either a primary amine, secondary amine, or an imine functional group (absorbances from 3200 to 3400 cm^{-1}) (3).

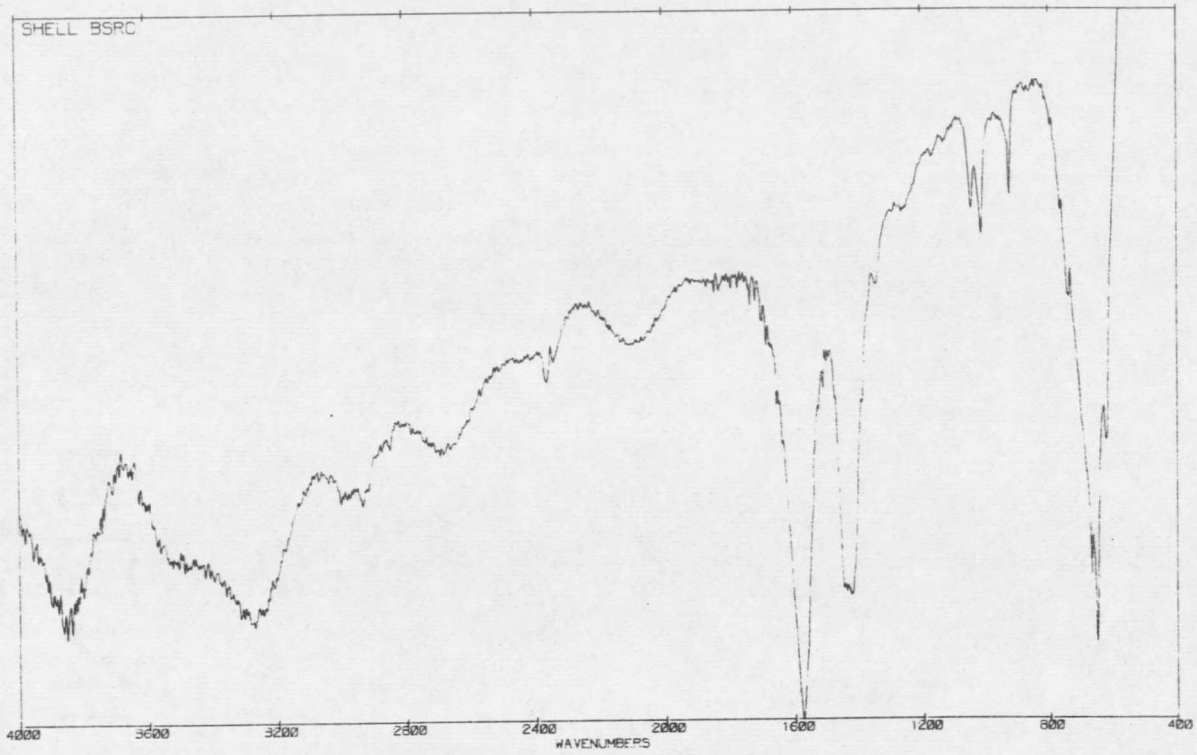


Figure 23. IR spectrum of MARS.

Appendix V

Glass Bead Breakage of *Candida albicans* and Other Medically Important Yeasts

The expense and tedium involved in using standard procedures to break fungal cells, such as the Sorvall Cell Fractionator and the Braun Mechanical Cell Homogenizer (6), have led researchers to seek alternative cell fractionation methods. Recently, investigators have found that several non-medically important fungi can be successfully broken using glass beads and vortex type mixers (5, 7, 10). Although Chaffin et al. used a similar method to break *Candida albicans* (1), the procedural details were not well defined. Reported here are specifics which will allow investigators to break medically important yeast cells in a simple, efficient, and inexpensive way.

Candida albicans strain 9938, *Candida tropicalis*, *Torulopsis glabrata*, *Cryptococcus nepformans*, *Saccharomyces cerevisia*, and *Sporothrix schenckii* (Montana State University, Bozeman, MT.) yeast cells were grown in glucose (2%)-yeast extract (0.3%)-peptone (1%) broth medium for 24 to 48 h at 37°C and 160 rpm(Incubator-Shaker, New Brunswick Scientific, Edison, N.J.), harvested

by centrifugation, and washed three times with saline. Under these conditions, greater than 99% of the cells of all organisms except S. schenckii cells were predominantly yeasts, but some mycelial clumps were present. The washed cells were centrifuged at 2000 x g for 30 min., and the supernatant liquid was discarded just prior to breaking the cells. The cells were kept on ice until use and were broken the same day of harvest.

Yeasts were fractionated using a technique similar to that reported by Van Etten and Freer for other fungi (10). The wet weight of packed cells, bead size, and vortexing time were varied to determine which set of conditions would yield the highest percentage of broken cells. In all cases, the cells were resuspended in 5 ml of 0.01 M phosphate buffered saline and transferred to a 30 ml heavy walled centrifuge tube (Corex tubes, Corning Glass Works, Corning, N.Y.). Ten grams of glass beads (Glasperlen B. Braun Instruments, San Francisc, CA.) were added to each tube, and the tubes were sealed with parafilm. The tubes were then alternately placed on a Vortex mixer (VWR Scientific, Seattle, WA.) at the highest setting for 15 s and cooled in ice water for 15 to 30 s. After the desired number of cycles the beads were removed by low speed

centrifugation (10. The results show that glass beads of 0.45-0.50 mm diameter (Table 21) and vortexing for a total of 90 s (Fig. 24) represent the best conditions for breaking C. albicans cells. Under these conditions, substantial cell breakage occurred over the entire range of cell concentrations tested (Table 21). Our observations also show that cell breakage is due mostly to small fractures in the cell wall rather than severe fragmentation (Fig. 24).

A high percentage of broken yeasts of C. neoformans, S. cerevisiae, and C. tropicalis was obtained using 0.45-0.50 mm beads (Table 21). Yeasts of S. schencki were fractured more efficiently using 0.25-0.30 mm glass beads, but even with these beads breakage was not as impressive as when other species were tested.

Hyphae of C.albicans were also brolen by this method. Hyphae were obtained by growing cells overnight in a synthetic medium at 37°C (Hazen and Cutler, manuscript in preparation). Under these conditions at least 85% of the cells were of hyphal form. Although quantitation was difficult to assess, it appeared that 5-20% fewer hyphae were broken than parent yeast cells.

There are advantages to using the method described

Table 21. Breakage of yeast cells under various conditions.

Organism	Wet wt of cells (mg)	Diameter of glass beads (mm)	Vortexing time (s)	Cell breakage (%)[2]
Candida albicans	200	0.17–0.18	30	<1
	200	0.17–0.18	60 or 90	1–5
	150	0.25–0.30	30 or 90	45–55
	200	0.25–0.30	30, 60, 90	35–45
	50 or 100	0.45–0.50	90	68–80
	200	0.45–0.50	30	45–60
	200	0.45–0.50	60	70–80
	200	0.45–0.50	90	90–98
	300	0.45–0.50	90	75–85
	1000	0.45–0.50	30	56
		0.45–0.50	60	77
		0.45–0.50	90	91
	2000	0.45–0.50	30	54
		0.45–0.50	60	76
		0.45–0.50	90	84
Torulopsis glabrata	500	0.45–0.50	90	78
Candida tropicalis	1000	0.45–0.50	90	92
Saccharomyces cerevisiae	500	0.45–0.50	90	94
Sporothrix schenckii	500	0.45–0.50	30	5.5
		0.45–0.50	60	14
		0.45–0.50	90	24.5
	500	0.25–0.30	30	32
		0.25–0.30	60	46
		0.25–0.30	90	68
	300	0.17–0.18	30	24.5
		0.17–0.18	60	45
		0.17–0.18	90	47
Cryptococcus neoformans[3]	100	0.45–0.50	30	20
		0.45–0.50	60	63
		0.45–0.50	90	84

[1] Fractionation was conducted by placing cells in 5 ml of buffer and adding 10 g of variously sized glass beads in a 30 ml Corex tube. Vortexing was done for different lengths of time at the maximum speed of the Vortex mixer.

[2] Percent cell breakage was determined by counting the number of 'ghost' cells in a population of 200 ghost and intact cells, dividing that number by 200 and multiplying by 100

[3] India ink negative staining indicated that the organism was encapsulated

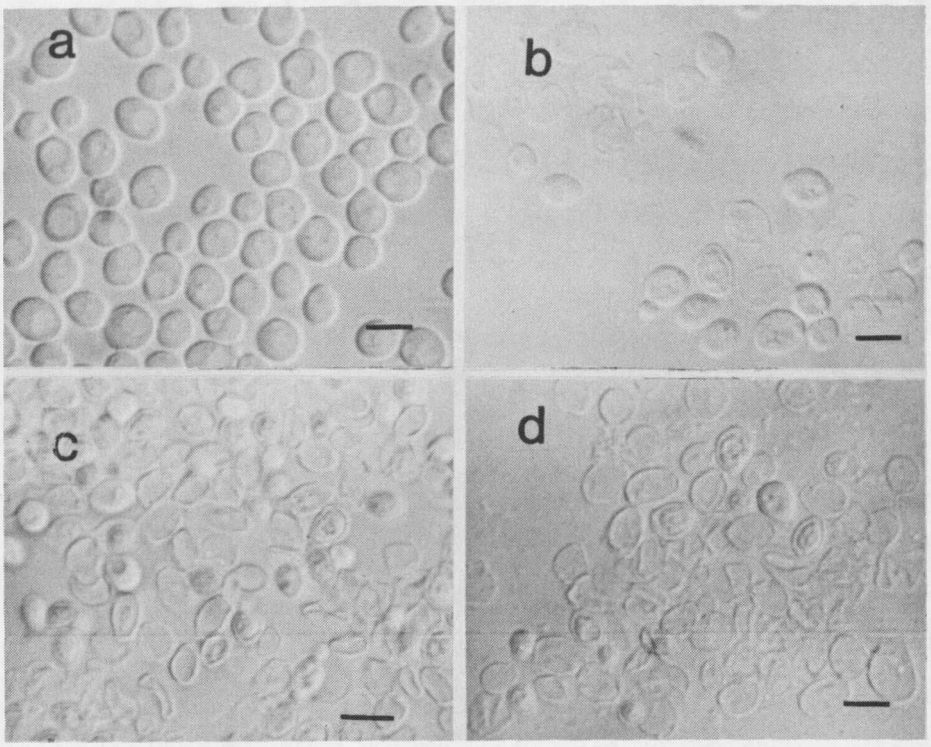


Figure 24. Effect of vortexing time on breakage of C. albicans yeast cells. Two hundred mg of packed, wet cells were suspended in 5 ml of buffer in a 30 ml Corex tube and 10 g of 0.45-0.50 mm diameter glass beads were added. Vortexing was done at the highest setting of a Vortex mixer. (A) represents yeast cells before fractionation, (B) represents yeast cells after 30 s exposure to vortexing, (C) after 60 s exposure, and (D) after 90 s. Bar represents 4 um.

above for breaking yeast cells. The procedure requires only 90 s of vortexing rather than 6 min (1). Heat generation during cell breakage, which can be difficult to control with the Braun homogenizer, is minimized by periods of cooling. Similar to the Braun homogenizer, all steps can be performed aseptically, but unlike the Braun (6), the resultant broken cells are less fragmented. Rather, the appearance of the cell walls resembles fractionated cell preparations obtained with the Sorvall Cell Fractionator (6). The Sorvall Cell Fractionator, however, is a cumbersome instrument which has the additional disadvantages of being expensive and difficult to sterilize.

LITERATURE CITED IN APPENDICES

1. Chaffin, W.L., R.A. Barton, E.L. Jacobson, and M.K. Jacobson. (1979). Nicotinamide adenine dinucleotide metabolism in Candida albicans. J. Bacteriol. 139: 883-888.

2. Chaffin, W.L. and S.J. Sogin. (1976). Germ tube formation from zonal rotor fractions of Candida albicans. J. Bacteriol. 126: 771-776.

3. Cross, A.D. and R.A. Jones. (1969). An Introduction to Practical Infra-red Spectroscopy, 3rd Edition. Butterworth and Co., Ltd., London.

4. Lee, K.L., H.R. Buckely, and C.C. Campbell. (1975). An amino acid liquid synthetic medium for mycelial and yeast forms of Candida albicans. Sabouraudia 13: 148-153.

5. Lipinski, C., A.J. Ferro, and D. Mills. (1976). Macromolecular synthesis in a mutant of Saccharomyces cerevisiae inhibited by S-adenosylmethionine. Molec. Gen. Genet. 144: 301-306.

6. Ribi, E. and K.C. Milner. (1967). Description of Bacteria and Preparation of Cell Walls, p. 13-28. In Meth. Immunol. Immunochem., vol. 1, Acad. Press, Inc., N.Y.

7. Richards, W.C. and S. Takai. (1979). A microfuge tube rechnique for disruptiing microquantities of fungal spores. Anal. Biochem. 100: 240-243.

8. Shepherd, M.G., Y.Y. Chiew, S.P. Ram, and P.A. Sullivan. (1980). Germ tube induction in Candida albicans. Can. J. Microbiol. 26: 21-26.

9. Soll, D.R. and G.W. Bedell. (1978). Bud formation and the inducibility of pseudo-mycelium outgrowth during release from stationary phase in Candida albicans. J. Gen. Microbiol. 108: 173-180.

10. Van Etten, J.L. and S.N. Freer. (1978). Simple procedure for disruption of fungal spores. Appl. Environ. Microbiol. 35: 622-623.