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Analysis of Factors Affecting Germ Tube Formation in Candida albicans

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ANALYSIS OF FACTORS AFFECTING GERM TUBE FORMATION IN *CANDIDA ALBICANS*

by

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VITA

Jordan Harry Pollack was born in Chicago, Illinois, on October 27, 1949, to Israel and Sara Pollack. He graduated from the Chicago Jewish Academy in June, 1966, and received a Bachelor of Arts degree from the University of Chicago, Division of Biological Sciences, in December, 1973.

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He is a co-author of the following publications:

Papers:

Hashimoto, T., J. H. Pollack and H. J. Blumenthal. 1978. Carotenogenesis associated with arthrosporulation of Trichophyton mentagrophytes. J. Bacteriol. 136: 1120-1126.

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ACES	N-[2-acetamido]-2-amino-ethanesulfonic acid
ADH	alcohol dehydrogenase
BS	buffered salts solution
BSA	bovine serum albumin
BSC	buffered salts, with bicarbonate solution
Chs	chitin synthase
conc.	concentration
dH_2O	distilled water
dia.	diameter
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethylsulfoxide
DTT	dithiothreitol
EDTA	ethylenediamine tetra-acetic acid
GABA	γ-amino butyric acid
Glc	glucose
GlcN	glucosamine
GlcNAc	N-acetylglucosamine
HEPES	N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid
LAA	Lee's amino acids
LM	Lee's medium
ManNAc	N-acetylmannosamine
MEM	modified Eagle medium
MES	2-[N-morpholino]ethane-sulfonic acid
MOPS	3-[N-morpholino]propane-sulfonic acid
PAGE	polyacrylamide gel electrophoresis
PIPES	1,4-piperazinediethane-sulfonic acid
Pro	proline
SATD	surface area to tube depth ratio
SD	standard deviation
SDA	Sabouraud dextrose agar
SDB	Sabouraud dextrose broth
SEM	standard error of the mean
TAPO	tris-1-aziridinyl-phosphine oxide
TAPS	tris(hydroxymethyl)-methylaminopropanesulfonic acid
TCA	tricarboxylic (or citric) acid cycle
TEM	transmission electron microscopy
Tris	tris[hydroxymethyl]-aminomethane
x g	times force of gravity

The following are the strains of Candida albicans mentioned in this study:

CA2	A72
ATCC 10261	CM45348
ATCC 58716	h0G301
ATCC 14053	

CHAPTER I

GENERAL INTRODUCTION

Statement of Problem

Candida albicans is an opportunistic fungal pathogen, found mostly in warm-blooded animals, and which is transmitted by animal to animal contact. C. albicans has long been known to be capable of infecting virtually every tissue of the body, as can be attested by the more than 100 synonyms for this organism (Van Uden and Buckley, 1970; Odds, 1979). Most of these infections are superficial infections of the mucous membranes of the mouth or vagina, and, to a lesser extent, the skin. However, with the increased usage of broad spectrum antibiotic and immune suppressive therapy, deep seated infections have become more common. Other predisposing conditions of candidosis include infancy, pregnancy, diet, oral contraceptives, trauma, occlusion with warmth and humidity, diabetes mellitus, and natural immune deficiencies. These conditions suggest a number of general areas for studying C. albicans pathogenicity, namely: environmental, nutritional, physio-chemical, hormonal and immunological factors (Ryley, 1986).

This dissertation will deal mainly with environmental and nutritional factors affecting the transition of C. albicans from growing as a budding yeast to growing as an elongating hyphae. It is still debatable whether the transformation from the yeast to mycelial form of growth is necessary for infection to occur, or whether it is a consequence of growth in tissues. However, the fact that mycelia are observed in almost all infections has led many to suggest that understanding the dimorphic process would also lead to

a better understanding of the disease process. Notwithstanding this desire to better understand the disease process in C. albicans, the study of the dimorphic transition is interesting in its own right and as a possible model for understanding transformations of other cell types. Furthermore, since this transformation is induced by simple chemical compounds it serves as another example of a system where a message is transmitted by a biological signal.

The dimorphic nature of C. albicans was first noted by Grawitz in 1877. Audrey in 1887 proved the yeast and mycelial forms were two morphogenic forms of the same organism whose interconversion depended on the growth conditions. While a number of physical and environmental factors affecting hyphal formation had been studied for many years, the study of nutritional factors was hampered by the time required to obtain hyphae, which was generally a day or more. Thus, until McClary (1952) showed otherwise, the general consensus was that hyphal formation was the result of poor nutrition. Subsequently it was demonstrated by a number of researchers that C. albicans could be rapidly induced to form short hyphae, or germ tubes, in serum (Reynolds and Braude, 1956; Taschdjian et al., 1961; Mackenzie, 1962). The search to find which factors in serum were responsible for the rapid germ tube formation, plus the later discovery that mycelial formation was accompanied by an increase in chitin formation (Braun and Calderone, 1978; Chiew et al., 1980), led to findings that a number of small molecules such as certain amino acids and N-acetyl-D-glucosamine (GlcNAc, the monomer of which chitin is composed) could induce germ tube formation. Despite the use of these simplified, defined media, there still remained a number of conflicting reports as to the effect of various physical environmental factors and nutritional additives on germ tube formation. Some of these

conflicting results, especially the role of pH, temperature, and glucose will be addressed in this dissertation with the goal of explaining the reasons for these conflicts.

More recently Sullivan and Shepherd (1982) have proposed that a nitrogen-containing inducer need not be metabolized in order for germ tube formation to occur, as long as the cells are supplied with a usable carbon source. This idea of a non-metabolized inducer is appealing for it is a mechanism proposed for other biological systems in which small molecules serve as signals, such as bacterial spore germination and nervous system transmissions. This thesis will examine this finding as to its reproducibility and also its significance in light of the discovery by the author that ethanol is a competent inducer of germ tube formation even in the absence of an exogenous nitrogen source.

The following review of literature will first examine in detail previous approaches to the study of dimorphism in C. albicans.

Review of the Literature

Biology of C. albicans

Although C. albicans is generally referred to as a dimorphic fungus, in reality it grows in a number of forms. The growth form usually encountered on standard laboratory media, such as Sabouraud dextrose agar, is the yeast form, which reproduces by budding. The yeast cells, commonly referred to as blastoconidia, are ovoid to spherical in shape ranging in size form 2 x 3 to 6 x 10 μm (Odds, 1985). In contrast to the saprophytic form, in infected tissues a variety of forms can be detected. Besides numerous yeasts one can also find true hyphae and what are called pseudohyphae. Hyphae, which have

a thin cylindrical appearance, grow by elongation and have septa at regular intervals. They arise either by branching from preexisting hyphae or by evagination, popularly called "germination," from yeast cells. Pseudohyphae, on the other hand, are essentially elongated yeast cells which grow by budding, although other differences have been noted (Yokoyama and Takeo, 1983). However, unlike yeast cells, the buds fail to separate from the mother cell, thereby forming a filamentous looking chain, which in many instances is difficult to distinguish from true hyphae, except for constrictions at the mother/daughter cell junction, instead of septa. The initial evaginations of hyphal elements form the yeast cells, called "germ tubes" or "mycelial sprouts," (Rippon, 1982) are very difficult to distinguish from a pseudohyphal bud unless the constriction between the two cells is obvious, which is not always the case. Finally, C. albicans at 25°C, under starvation growth conditions, will form chlamydospores, highly refractile spores which may be a "dormant" or "storage" stage of this fungus (Odds, 1985). They are generally found associated with hyphal or pseudohyphal elements and attached to the filaments by means of "suspensor cells."

The terms "germination" and "germ tube" used in conjunction with the initiation of hyphal growth from yeast cells is due to the apparent similarity between this process and true spore germination, as can be seen by the use of the term "blastospore" to describe the yeast cells. As the yeast cells are not dormant cells, and as the ontogeny of spore germination at the ultrastructural level is different from that of hyphal evagination in C. albicans, the terms are really misnomers. As suggested by Odds (1985), since the term germ tube is almost universal in usage, it will be retained; however, the phrase "germ tube formation" will be used instead of "germination."

Significance of Germ Tube Formation in Candida albicans

Prior to the discovery of the effect of serum on C. albicans in 1954 (Hu et al., 1954; Johnson, 1954) and the subsequent adoption of the germ tube test for identification of this yeast (Reynolds and Braude, 1956; Taschdjian et al., 1960), the organism was identified primarily by its ability to form chlamydospores, usually on corn meal agar lacking glucose, and by its sugar fermentation pattern (Skinner, 1947). Chlamydospore formation is almost unique to Candida albicans, although Candida stellatoidea, Candida tropicalis and Candida clausenii will also form some chlamydospores especially in the presence of Tween 80 (Odds, 1979; Walker et al., 1960; Difco Technical Information, 1976, "Detection and identification of Candida albicans," pp. 1-10). C. albicans is also the only yeast species to ferment (gas production) glucose, galactose, and maltose, but not lactose or sucrose (Skinner, 1947; Odds, 1979). These tests are not rapid, taking a minimum of one day. Interestingly, chlamydospore agars produce not only chlamydospores but also pseudomycelia in almost all species of Candida (Odds, 1979). This last fact may help explain why although true hyphae are produced only by C. albicans and some strains of C. stellatoidea (Rippon, 1982), the significance of using germ tube production in identifying C. albicans was probably not considered initially. As recently as 1964, Winner and Hurley in their important monograph on C. albicans, after distinguishing between true and pseudomycelia, concluded that:

> ...it is difficult to draw hard and fast distinctions between elongated yeast cells and pseudomycelium on the one hand and pseudomycelium and 'true' mycelium on the other. For practical purposes either of the terms seem to be permissible (p. 26).

They therefore used the term mycelium to describe both pseudo- and true mycelium. This unfortunate choice not to distinguish between the two growth forms persists in the literature to this day and is the source of much con-

fusion. It should be mentioned that although chlamydospore media are indeed capable of producing real hyphae in C. albicans (Schaar et al., 1974; Beheshti et al., 1975; Odds, 1985), perhaps because they were developed primarily for chlamydospore production, which is optimal at 25°C (Torosantucci and Cassone, 1983), their ability to produce true mycelia at 37°C may have been overlooked.

With the introduction of serum as a rapid (2 to 3 h) and highly effective means of producing germ tubes (with close to 100% of the cells forming germ tubes), a new era in the study of C. albicans was opened. Many attempts were made at explaining the mechanism of germ tube elicitation. Germ tube production, besides being just another means of identifying C. albicans, seemed to present a path to understanding the pathogenicity of C. albicans. This was because germ tube formation "is the only property that correlates with the observation of C. albicans as the most common yeast pathogen (Odds, 1979, p. 29)." The general logic behind this idea has been that hyphae provide the means by which the organism can penetrate tissues. The fact that opportunistic yeasts such as Candida (formerly Torulopsis) glabrata, which cannot form even pseudomycelium, and other dimorphic fungi such as Histoplasma capsulatum, Blastomycoses dermatitidis, and Paracoccidioides brasiliensis grow as hyphae saprophytically but as yeast in tissues, would tend to argue against this logic. On the other hand a number of experiments have shown that the ability of C. albicans to form hyphae is essential to its capacity to cause disease.

Odds (1979, p. 30) in a review on the subject argues that in vivo yeast and pseudohyphal forms exist alongside hyphae. In addition, studies showing the infectivity of the yeast and hyphal forms are contradictory.

Some find no difference in infectivity when injecting a number of hosts (mice, rats, rabbits or humans) with either yeast or mycelia by a variety of routes (Dastidar et al., 1971; O'Grady et al., 1967; Russell and Jones, 1973; Strippoli and Simonetti, 1973), while other studies showed that injecting the yeast form instead of mycelia resulted in greater pathogenicity (Mankowski, 1963; Mardon et al., 1975; Simonetti and Strippoli, 1973). Therefore, Odds (1979) discounts the mere finding of mycelia in infectious lesions or studies involving inoculating mycelia or yeasts as a means of determining the greater pathogenicity of either growth form. However, Odds (1979) does consider mycelium formation a factor in the disease process based on the following considerations:

a) In studies on oral thrush (Taschdjian and Kozinn, 1957), Candida diarrhea in infants (Kozinn and Taschdjian, 1962) and in a study on denture stomatitis (Budtz-Jorgensen et al., 1975), hyphal forms were detected exclusively, or to a much greater extent, only when lesions were evident. Direct smears of asymptomatic controls showed the presence primarily of C. albicans yeast forms;

b) The species of Candida most able to kill different cell types in culture was C. albicans, followed by C. stellatoidea and C. tropicalis. These species are capable of producing filaments. Non-filament producing species show few pathologic effects (Hurley and Stanley, 1969; Howlett, 1976; Louria and Brayton, 1964; Stanley and Hurley, 1967, 1969); and

c) Mutants of C. albicans resistant to polyene antifungal drugs, which have been shown to have a reduced ability to form filaments, also have a diminished virulence (Athar, 1971; Hamilton-Miller, 1972; Hebeka and Solotorovsky, 1965; Lones and Peacock, 1959).

Odds (1979), therefore, concludes that while there is probably an association between ability to form germ tubes and virulence, that it is not an absolute requirement. In other words, "...it would be incorrect to suggest that mycelial and blastospore forms of C. albicans are respectively pathogenic and non-pathogenic or invasive and non-invasive (p. 198)."

Louria (1985), in another review on the subject, is more emphatic in stating that the ability to form hyphal elements is critical to the initiation of tissue involvement. In support he cites other papers by some of the same groups cited by Odds (Hurley and Winner, 1963; Louria et al., 1963; Louria and Brayton, 1964; Taschdjian and Kozinn, 1957). He makes the point that the difficulty of various groups in demonstrating lethality of mycelial inocula may be an "anatomical artifact" (p. 34), since the mycelia, being large and awkward, may not be able to get out of the intravascular compartment and hence are subject to killing by polymorphonuclear leukocytes and mononuclear cells. He further argues that a study by Richardson and Smith (1981) is persuasive evidence that the formation of germ tubes is a "virtual prerequisite for pathogenicity" (p. 35). This study showed that strains of Candida albicans virulent for mice, formed more germ tubes than attenuated strains in mouse or human sera or in mouse kidney extracts.

Other recent papers dealing with the pathogenicity of C. albicans do not resolve the issue, but, in general, do support the view that the ability to form germ tubes is an important virulence factor, if not an absolute requirement for pathogenicity. The least supportive of this statement is a paper by Shepherd (1985), in which a strain of C. albicans incapable of converting to the hyphal form (CA2) was shown to be as capable of killing mice as the germ-tube forming strains (ATCC 10261, CMI45348 or A72). He seemed to

suggest that nonpathogenicity of some strains may be due instead to their slow growth since a number of auxotrophic strains, whose growth in infected animals was assumed to be impaired, were non-pathogenic, while a prototrophic, tetraploid strain constructed from two of the auxotrophic strains was pathogenic. He did show that a mycelial mutant, incapable of converting to the yeast form (hOG301; it does appear to form pseudohyphal elements, though, Hubbard et al., 1986), and hence, by inference, mycelia, in general, are capable of causing lethal infections in mice if they are inoculated intravenously in sufficiently high concentrations. However, the mice inoculated with the mycelial mutant were found to have 2- to 3- fold greater survival times than those inoculated with a 10-fold smaller mass of the wild type yeast cells. This strain, developed by Poulter (Hubbard et al. 1986), inoculated intraperitoneally, as well as an earlier mycelial mutant (MacKinnon, 1940), had been reported to be nonpathogenic. Thus, Shepherd's report, while supporting the pathogenic capacity of the mycelial form demonstrates that previous reports of reduced or non-pathogenicity of the mycelial form, are possibly technically related.

Other recent groups, generally working with mutants, obtained different results. Sobel et al. (1984), studying a variant strain incapable of forming hyphae at 37°C, but capable of doing so at 25°C, found that it adhered significantly less to epithelial cells than cells from strains capable of forming germ tubes, was less likely to initiate vaginal colonization in rats and infections, once established, were often transient. They concluded that germ tube formation was an important virulence factor, but not absolutely essential unless the cells were totally incapable of forming germ tubes. Klotz et al. (1983) showed that yeast cells, which adhered to viable porcine vascular tissue

in tissue culture medium, were able to penetrate the endothelium before germ tubes were produced. They, nevertheless, concluded that the ability to form germ tubes probably potentiates the process. It should be noted, though, that Rotrosen et al. (1985) dispute the claim that yeast cells alone can penetrate the endothelium. Kinsman and Collard (1986) found that subculturing a virulent strain of C. albicans led to a significantly reduced ability to form germ tubes (90% vs. 17%) which correlated with its reduced virulence. Furthermore, they found that treating ovariectomized rats with estrogen, which promoted C. albicans infection, also resulted in generally longer germ tubes which penetrated the epithelium. In rats treated with progesterone, on the other hand, the germ tubes were clumped, leukocytes were present, and the cells were cleared. These results were advanced as support for the idea of germ tube formation being an important virulence factor. The evidence which supports the role of the hyphae most is that of Martin et al. (1984). They showed that rats fitted with a palatal acrylic appliance could be infected only by strains of C. albicans capable of forming true hyphae. Furthermore, neither their germ-tube-negative strain, nor a strain capable of forming only pseudohyphae, could produce any marked histological changes in the oral epithelium.

Other papers dealing with this controversy are Bistoni et al., (1986), Gresham and Whittle (1961), and Saltarelli et al. (1975), which support the idea of greater pathogenicity of the hyphal form, and Mardon et al. (1975) and Evans (1980), which maintain that the yeast form is more pathogenic.

After considering all of the evidence, it is possible to surmise that since the infection of every tissue may depend on a number of different factors, that it is very likely that for some tissues germ tube formation may be required. Thus when pathogenicity is measured by the death of a mouse,

it appears that in many cases hyphal formation is not required. On the other hand, those experiments which measure pathogenicity by the ability to infect a mucous membrane apparently show that hyphal formation does play a role.

Regardless of the actual role that hyphal formation plays in infections of C. albicans, it is important to realize that C. albicans is just one of a number of dimorphic fungi which changes its form in the pathogenic state. Although they do not all have the same requirements to convert from one state to the other, many of the conditions are the same as will be discussed in greater detail below (for reviews see Bemmann, 1981; Cole and Nozawa, 1981; San-Blas and San-Blas, 1984; Rippon, 1980; Szaniszlo et al.,1983; Cutler and Hazen, 1983; Dabrowa and Howard, 1983). Since, C. albicans is perhaps the easiest of these organisms to grow, it also serves as a useful model for the understanding of the dimorphic process.

Perhaps even more intriguing is the possibility that the induction of germ tube formation in C. albicans may be just one of many instances where a biological signal is mediated either by a small molecule or by a change in the environment. A number of amino acids, in particular γ-amino butyric acid (GABA), proline, glycine, β-alanine, taurine, L-glutamate, and L-aspartate, have been shown to act as neurotransmitters (Usherwood, 1978; Fagg and Foster, 1983; Iverson, 1984). Similarly, glucose, alanine, proline, adenosine, and a number of their analogs, can induce bacterial spore germination (Scott and Ellar, 1978; Rossignol and Vary, 1979; Szulmajster, 1979). Changes in pH are reported to induce developmental changes in sea urchin (Suprenant and Marsh, 1987) and shrimp eggs (Hand and Carpenter, 1986), besides fungi (Roure and Bouillant, 1986).

Approaches to the Study of Dimorphism in C. albicans

It is possible to group almost all studies of C. albicans dimorphism, and probably fungal dimorphism in general, into two general approaches. One, is the determination of qualitative or quantitative differences in some subcellular fraction of the different morphological forms. These differences generally lead the researcher to conclude that the dimorphic transition is "caused" by the changes in the subcellular fraction under consideration and to propose some mechanism by which these differences lead to the dimorphic transition. The second approach is generally a survey of substances which are either capable of inducing or interfering with the dimorphic transition; the types of compounds responsible also leading to speculation on the mechanism of dimorphism. Both of these approaches may be considered elementary since they are either just descriptive of, or dependent on the final and the initial states of the organism. Thus, the mechanisms they propose for dimorphism can be no more than speculative. However, the fact that no clear picture has emerged from numerous studies utilizing these approaches, makes it more understandable why more sophisticated approaches, using genetics or studying the dynamics of dimorphic change, have been so hard to develop. What is known about dimorphism in C. albicans will now be examined more closely.

A. Differences Between the Yeast and Hyphal Forms of C. albicans.

1. Chemical Composition of the Cell Walls.

Although differences in a number of subcellular fractions have been investigated, the cell wall has been, perhaps, the most studied. This is not surprising, since the cell wall is considered to be the ultimate determinant of cell shape (Bartnicki-Garcia, 1968; Bartnicki-Garcia, 1973; Brody, 1973; Mishra,

1977). The evidence that cell walls may also play a major role in C. albicans dimorphism comes from both ultrastructural, as well as chemical, studies.

The cell wall composition of C. albicans has been analyzed in a few studies. Like most fungi, C. albicans walls consist mostly of polymers of glucose (glucan), mannose (mannan or mannoprotein), N-acetylglucosamine (chitin), with small amounts of protein and lipid (Kessler and Nickerson, 1959; Bishop et al., 1960; Chattaway et al., 1968). There have also been isolated reports of the presence of fucose, arabinose, galactose, uronic acid, and N-acetylgalactosamine (Stoddart and Hebertson, 1978; Ray et al., 1979; Gopal et al., 1984b, 1984c)

Chattaway et al. (1968) reported that the walls of the hyphal and yeast forms of C. albicans were qualitatively similar. However, they found a significant difference in the amount of chitin (1.5% of the dry weight of the yeast wall, 6.0% of the hyphal wall), which were independent of the growth temperature and the culture conditions. In addition, 39.2% of the alkali insoluble fraction of the yeast form was released by β-glucanase while only 18.8% was released from the corresponding hyphal fraction. The percent of protein in the alkali insoluble residue was three- to four-times greater in the yeast form than the hyphal form, and there were considerable differences in the amino acid composition of this same fraction between the two forms. Histidine was present (11.2 μmol % of the amino acids in alkali insoluble fraction) but arginine was absent in the yeast form fraction, while arginine was present (5.3 μmol %) and histidine was lacking in the same fraction of the hyphal form. These results indicated that there were differences in the tertiary structure of the polysaccharide-protein complex of the two forms.

Yamaguchi (1974a, 1974b) reported that the hyphal form had 20% less

mannan than the yeast form, but 50-60% more alkali-insoluble glucan when hyphal growth was controlled by reducing the level of biotin. Both of these results conflict with those reported by Chattaway et al. (1968). Schwartz and Larsh (1980), on the other hand, found the yeast form to contain more mannan (22.3% versus 16.7%), but less total glucan (63.0% versus 76.5%) and chitin (6.7% versus 15.8%) than the hyphal form.

Mattia et al. (1982) demonstrated that during germ tube formation induced by GlcNAc, there was a 10% increase in total protein during the first 150 min, followed by a 30% drop in the next 120 min. Since the total polysaccharide content continuously increased during the same period, this translated into an inverse relationship with respect to dry weight. The percentage protein in the wall decreased from 37 to 18% while the percentage of polysaccharide increased from 30 to 40% over a 270 min period. Chitin was the only wall polysaccharide found to increase its relative value (from 1.6 to 8.4% of total wall saccharide). Mannan decreased from 25 to 18.7%, while glucan remained constant at approximately 73%.

Elorza et al. (1983a) measured the incorporation of labelled glucose into chitin, mannan and glucan over a 5 hour period. By this method, they determined mannan to be a much larger component of the yeast wall (19%) than the hyphal wall (4%), while much more chitin was present in the hyphal form (21%) than the yeast form (9%). They found little difference in the incorporation of glucose into glucan (approximately 70% in both forms). The greater importance of chitin to the dimorphic process was shown by the fact that when cells grown at 28°C (yeast form) or 37°C (hyphal form) were shifted to the opposite temperature, there was no change in the kinetics of either mannan or glucan incorporation. However, chitin synthesis of cells originally

grown at 28°C, increased rapidly when the cells were transferred to 37°C. It should be mentioned, though, that this effect may have been totally due to the increase in temperature since the yeast morphology did not change.

Sullivan et al. (1983) found little difference in the composition of yeast and hyphal phase glucans and mannans. The insignificant increases in the relative ratio of these cell components seemed to be associated with growth or starvation rather than dimorphic transition. On the other hand, the chitin fraction of the cell wall increased from 0.6% to 2.7% (w/w) during the 3 h germ tube induction period. Comparison of incorporation of radiolabelled glucose into glucosamine also showed that 10 times more glucose was incorporated in germ tube forming cells than in yeast growing controls.

The glucans have been determined by analysis of both the cell walls and of the fibers formed from regenerating spheroplasts to be β(1->3) and β(1->6) chains, without mixed intrachain β(1->3) and β(1->6) linkages (Bishop et al., 1960; Yu et al., 1967; Gopal et al., 1984b, 1984c; Lyon and Domer, 1985). Gopal et al. (1984a) compared the relative concentrations of the glucans of the yeast and hyphal cells. They found the alkali- and acid-insoluble glucan of both the yeast and hyphal forms to consist of approximately 30% β(1->3)-linked residues and 50% β(1->6)-linked residues, the remaining glucan being either non-reducing end groups or branch points. They concluded, therefore, that morphology is not determined simply by the ratio of β(1->3) to β(1->6) linkages. However, they reported that in germ tube forming cells (the time when this was measured was not indicated) β(1->3) glucan made up 67% of the insoluble glucan. Since germ tube forming cells still had the mother cells attached, they surmised that during the morphogenic transition, β(1->3) glucan was synthesized exclusively. This would be analogous to what

occurs in regenerating spheroplasts, which also synthesize mainly β(1->3) glucan (Kreger and Kopecká, 1975; Gopal et al., 1984c). In addition, they noted that as the cell formed germ tubes, the insoluble glucans were more branched. Branch points accounted for 6.7%, 12.3% and 17.4% of the residues in the alkali- and acid-insoluble glucan of the yeast, germ tube forming, and hyphal cells, respectively. The acid-soluble and the alkali-soluble glucans of both the hyphal and yeast forms contained approximately 70% β(1->6) glucan, and were not thought to play a role in morphogenesis.

Some recent studies have also found differences in the mannans and mannoproteins between the yeast and hyphal forms of C. albicans (Elorza et al., 1985; Milewski, et al., 1986; Shibata et al., 1986). While there is an indication that there is suppression of mannan synthesis during the yeast to hyphal transformation, none of the authors claimed that this change was a "cause" of dimorphism. These changes in the mannoprotein may be means of identifying specific markers for hyphal formation, and they may be useful in the diagnosis of candidiasis (Smail and Jones, 1984; Sundstrom and Kenny, 1984; Ponton and Jones, 1986).

2. Electron Microscopic Localization of Wall Polymers.

The chemical differences in the structure of the cell wall have been visualized with the use of transmission electron microscopy (TEM). The yeast phase cells, viewed by TEM are reported to be multilayered. Most researchers consider it to consist of five layers of different electron density (Cassone et el., 1973; Diaczenko and Cassone, 1972; Drewe, 1981; Howlett and Squier, 1980; Hubbard et al., 1985; Persi and Burnham, 1981). Although one group reported as many eight layers (Poulain et al., 1978, 1981), this report is

eclectic, consisting of a compilation of results from different growth and staining conditions, and the number of layers may be exaggerated (Szaniszlo et al., 1983; Odds, 1985).

Cassone et al. (1973) demonstrated that the material of the electron transparent "fourth" layer, appeared to accumulate during germ tube formation until it became the main component of the germ tube wall. In order for the electron transparent layer to emerge, the fibrous components of the "third" layer seemed to open up, and the two outermost layers were progressively lysed. However, as the hyphae matured, the outer components were progressively resynthesized. Thus, the appearance of the hyphal wall was similar to the yeast wall in that it consisted of five layers. It was different in that it was only 120-135 nm thick, about half the thickness of the yeast wall, and in that the electron transparent layer remained the dominant layer. Based on studies of other fungi, the electron transparent layer was assumed to consist mostly of chitin, which seemed to correlate with the increase in this polymer during germ tube formation, detected by Chattaway et al. (1968).

Scherwitz et al. (1978), using lithium permanganate fixed cells, instead of acrolein, TAPO (tris-1-aziridinyl-phosphine oxide) and osmium tetroxide fixed cells used by Cassone et al. (1973), concurred that the electron transparent fourth layer, was the major component of the germ tube wall. However, contrary to Cassone et al. (1973), they found that the third layer did not grow in the germ tube wall at all, while the electron dense second layer continued growing unchanged in structural density and thickness. They also showed that the septum of a "true" hypha, as opposed to the bud scar of a yeast or pseudomycelium, to contain a single, central pore. The septum itself was wider (170-220 nm) than the adjacent cell wall (120-160 nm) and con-

tained a narrow (20 nm) electron transparent band in the center which was connected with the electron transparent fourth layer of the hyphal wall. Howlett and Squier (1980), using glutaraldehyde and osmium fixed cells, seemed to confirm the results of Scherwitz et al. (1978). Hubbard et al. (1985), while also confirming the importance of the electron lucent layer to the germ tube wall, also suggested that the outermost, fibrillar border (layer 1?) was more apparent in the germ tube walls than in the yeast walls, and might also be important in their formation.

A few groups have attempted to correlate the electron microscopic appearance with the chemical composition of the different layers. Chattaway et al. (1976) demonstrated that dithiothreitol (DTT) caused the outer-electron dense layers of both the yeast and hyphal forms to appear more diffuse. The assumption was that DTT reduced disulfide bonds and that the outer layers probably consisted of glycoprotein. DTT, protease, and β(1->3) glucanase treatment led to the complete removal of the outer layers of the yeast form, but only partial degradation of the inner electron lucid layers. The resultant cells were osmotically fragile. Hyphal cells treated similarly were not fragile and retained their shape. To obtain protoplasts, from both the yeast and hyphal forms of C. albicans, it was necessary to add chitinase to the DTT, protease, and β(1->3) glucanase treatment (see also Domanski and Miller, 1968; Torres-Bauza and Riggsby, 1980). Thus, the chitin containing fraction was adjacent to the cell membrane.

3. Changes in Chitin and Glucan Synthases.

The chemical and ultrastructural studies mentioned above, generally agreed that the most obvious wall changes occurred in the chitin fraction.

It should be mentioned, though, that the electron lucidity may also be associated with changes in β(1->3) glucan. Both the chitin and glucan synthases of C. albicans have been studied.

Braun and Calderone (1978) demonstrated that the chitin synthase activity of hyphae (induced with $(NH_4)_2SO_4$ and glucose) was two times greater than that of yeast cells (grown on Sabouraud dextrose broth). This result correlated with 10-fold more labelled GlcNAc being incorporated into the acid-alkali-insoluble (chitin) fraction of hyphal growing cells than into yeast cells. Chiew et al. (1980) and Ram et al. (1983) found the expressed chitin synthase activity to increase 4- to 5-times during germ tube induction. Chiew et al. (1980) were not able to demonstrate inhibition of germ tube formation by polyoxin D, a chitin synthase inhibitor, (Endo and Misato, 1969; Endo et al., 1970) using micromolar concentrations. Becker et al. (1983) and Hilenski et al. (1986), however, were able to show that millimolar amounts were required for morphological alterations to occur. Ergosterol was also found to inhibit both chitin synthase and germ tube formation (Pesti et al., 1981; Chiew et al., 1982). However, both methanol and ethanol, which stimulated chitin synthase activity, inhibited germ tube formation (Chiew et al., 1982). Calcofluor white, which interferes with chitin deposition external to the plasma membrane, but not with synthesis in vivo (Herth, 1980), causes wall thickening and abnormal deposition of wall material, or bulges, especially in the septal region, but no inhibition of germ tube formation (Elorza et al., 1983b; Rico et al., 1985). Assuming that sufficient calcofluor white was used to totally inhibit polymerization, it would appear that whatever role chitin synthesis serves in germ tube formation is accomplished before the chitin is extruded to the outside, where it is polymerized. This would seem to be

highly unlikely. In fact, Gow and Gooday (1983) did not find any differences in the ultrastructure of chitin fibrils of the yeast and hyphal forms of C. albicans, both being of granular appearance, similar to those found in S. cerevisiae.

Since chitin synthase exists as a zymogen, requiring trypsin activation, some groups have proposed that differences in the activation state of the enzyme might be responsible for dimorphism (Ruiz-Herrera and Bartnicki-Garcia, 1976; Shearer and Larsh, 1985). Although a number of studies have looked at the zymogenicity of chitin synthase from C. albicans, none has shown any differences in the state of activation of the enzyme between the two phases (Braun and Calderone, 1979; Hardy and Gooday, 1983; Gozalbo et al., 1985). A more recent proposal is based on the finding that there are actually two chitin synthases. Sburlati and Cabib (1986) proposed that chitin synthase 1 (Chs 1) is involved in synthesizing lateral wall chitin, while the second form synthesizes mainly chitin of the bud region. Budding yeast cells may have lost the ability to activate the Chs 1 zymogen and thereby form lateral walls. Whether these two forms of chitin synthase exist in C. albicans, or in other dimorphic fungi, must still be verified.

Orlean (1982) also found that the specific activity of $\beta(1\rightarrow3)$ glucan synthase was consistently higher in 3 h filamentous cultures, which suggested its possibly involvement in dimorphism. On the other hand, there was no significant difference in the specific activities during the first 90 min of budding or filamentous growth. However, since this enzyme requires activation by nucleoside triphosphates (Szaniszlo et al., 1985), the actual conditions in vivo which control the spatial localization of this polymer remain to be investigated.

4. Differences in Lipid Composition

The lipid content of C. albicans varies between 3 and 30% of the dry weight of the cells (Bianchi, 1966; Yamaguchi, 1974; Sundaram et al., 1981). Between 80 and 90% of these lipids are phospholipids and sterols (Davies and Dening, 1972; Ballman and Chaffin, 1979). Although Ballman and Chaffin (1979) found no significant differences between the neutral and phospholipids of the yeast and mycelial phases, Sundaram et al. (1981) found the total lipid to decrease from approximately 20% to 11% following germ tube formation. Marriott (1975) reported that sphingolipids, phosphatidyl inositol, and phosphatidyl serine were found only in yeast membranes, while Sundaram et al. (1981) found no differences in the last two compounds and decreasing amounts of the first as germ tube formation proceeded. On the other hand, they did find the large decreases in the total phospholipid and sterol content when starved cells were induced to form germ tubes. However, the final concentrations of these fractions were only slightly different than growing yeast cells. Sekiya and Nozawa (1983) also showed that, based on electron microscopic visualization of membranes treated with the sterol-specific antibiotic, filipin, ergosterol was virtually absent from the region between daughter and parent during budding, while ergosterol was heterogeneously distributed on the plasma membrane during germ tube emergence. Shimokawa et al., (1986) have also demonstrated the importance of ergosterol to hyphal formation. They isolated mutants which were blocked in ergosterol synthesis and which accumulated 14-methyl sterols instead. These mutants could not form hyphae. They found similar inhibition of germ tube formation when the cells were treated with clotrimazole, an antifungal antibiotic, which also blocks ergosterol synthesis, but which does affect yeast growth. Hoberg et al. (1983) and

Braun et al. (1987) have also shown that cerulenin, an inhibitor of lipid biosynthesis, and sodium butyrate, a fatty acid, both block germ tube formation. They found that these compounds interfere with chitin synthesis, measured by labelled GlcNAc uptake into the acid- and alkali- insoluble chitin fraction, but not when measured using flow cytometry, which does not require harsh extraction. They suggested that these compounds may affect the ability of the cells to aggregate the chitin microfibrils correctly, or to affect membrane fluidity, and thereby affect chitin transport across the membrane. It is, thus, possible that the changes in lipid composition during dimorphism are another aspect of wall biogenesis (Sundaram et al., 1981).

5. Differences in Cytoplasmic Proteins.

The differences between the hyphal and yeast forms in other subcellular fractions have also been investigated.

Differences in the cytoplasmic proteins isolated from the two forms have been looked at by a number of investigators. Using polyacrylamide gel electrophoresis (PAGE), only one group has claimed to find a unique hyphal protein (Finney, et al., 1985). The majority of workers have found some qualitatively unique proteins in yeast phase cells but none in hyphal cells (Manning and Mitchell, 1980a, 1980b; Brown and Chaffin, 1981; Brummel and Soll, 1982; Ahrens, et al., 1983; Dabrowa and Howard, 1984; Sevilla and Odds, 1986a). Some proteins were quantitatively greater during germ tube formation, though. Thus, the preponderance of evidence, based on PAGE, is that no unique, detectable, proteins are synthesized during germ tube formation. Furthermore, it is not yet clear whether those proteins which are more abundant during the dimorphic transition are the result of environmental factors,

such as heat, rather than specific for germ tube formation to occur (Dabrowa and Howard, 1984; Sevilla and Odds, 1986a). On the other hand Dabrowa et al. (1970), using disc electrophoresis, and Syverson et al. (1975) and Mason and Smith (1986), using crossed immunoelectrophoresis (with rabbit antibody), have reported unique mycelial cytoplasmic proteins. Again, there was no attempt to differentiate between morphogenetic proteins and environmental factors. Angiolella et al. (1986) have recently claimed that modulations in protein patterns may be due to nutritional stress rather than a result of induction conditions. This was demonstrated by adding amino acids back to GlcNAc-induced cells, and showing the disappearance of the previously observed changes.

Singh and Datta (1979) concluded that both RNA and protein synthesis, but not DNA synthesis, are required for germ tube formation. Shepherd et al. (1980) and Oliver et al. (1982) have also reported that both the translational inhibitors cycloheximide, puromycin and trichodermin, and the transcriptional inhibitors, acridine orange, actinomycin D, and daunomycin, in concentrations which allow cell development, inhibited germ tube formation almost completely, but only if they were added during the first 15 min of induction. They concluded, therefore, that both mRNA and protein synthesis were inducible, and required for germ tube formation to occur. However, as with all inhibitor studies in living cells, it must be remembered that none are so specific as to inhibit only transcription or translation. All of the inhibitors used may also inhibit other systems (Shepherd et al., 1980). It would seem that a more synchronously growing population, and a more specific inducer, will be required in order to determine whether there are actually specific germ tube proteins synthesized, and if they exist, to identify them.

6. Changes in Cellular Metabolism

Another area that has been examined is changes in the cellular metabolism during germ tube formation. Chattaway et al. (1973) studied some enzymes involved in carbohydrate metabolism. They found that 6-phosphogluconate dehydrogenase and phosphomannose isomerase activity decreased in hyphal forming cells but increased in budding cells. Glutamine:D-fructose-6-phosphate aminotransferase, which may be a control point in chitin synthesis, showed a greater than doubling in activity during filamentation, but only a 20% increase in budding cells. After 4 hours, the activities in both cell types decreased. Although, the overall changes in phosphofructokinase activity were similar in both the hyphal and yeast forms, since it was more repressed in the hyphal phase, it was thought that this was sufficient to provide more fructose-6-phosphate for chitin and mannan synthesis. This was supported by the finding of higher concentration of fructose-6-phosphate, glucose-6-phosphate and ATP, an inhibitor of this enzyme, in the mycelial phase. Only 5% of the glucose in hyphal-forming cells used the hexose monophosphate (HMP) pathway versus 20% in yeast cells at the same time.

Land (1975a) found that whereas in yeast cultures (grown on NH_4Cl) large concentrations of acetate and pyruvate appeared early and remained constant for the first 60 min, none could be detected in germ tube forming cultures (induced with proline). In fact, the concentrations of all organic acids measured rapidly decreased 15 to 30 min after initiation of induction, and then the levels remained very low. In addition, they found most of the ^{14}C label from the inducer located in a fraction high in respiratory activity, which they assumed to be the mitochondria. They theorized that filamentation was associated with generation of reducing potential, and that a block in

electron flow led to hyphal morphology. They also showed that high glucose (10 mM) and low phosphate concentrations in their media seemed to favor filamentation. These conditions also were reported to contribute to mitochondrial repression, or the "Crabtree effect" (Crabtree, 1929; Koobs, 1972), which, in turn, might effect carbohydrate and cell wall biosynthesis, and hence morphology. They, therefore, further studied glucose metabolism and respiration (Land et al., 1975b). Yeast growing cells produced less ethanol and more CO_2 and also consumed more O_2 than germ tube forming cells. This supported their contention that hyphal formation involved a change from aerobic to fermentative metabolism. In addition, inhibitors of glycolysis, such as 2-deoxyglucose and iodoacetate, inhibited hyphal formation while oxidized methylene blue, an electron acceptor, was the most potent inhibitor. Mitochondrial inhibitors, however, were also inhibitory (Land et al., 1975b).

Schwartz and Larsh (1982) induced C. albicans to form germ tubes in tissue culture medium, instead of using proline or NH_4Cl and glucose as Land et al. (1975a) did. They could not detect any alcohol dehydrogenase activity in either form and therefore concluded that no Crabtree effect was involved, and that "metabolism is aerobically manifested, at least during log-phase, balanced growth conditions." They also found that metabolism through the hexose monophosphate pathway was greater in yeast cells than in hyphal cells. Franzblau and Sinclair (1983) also described C. albicans as Crabtree-negative. Aoki and Ito-Kuwa (1982) demonstrated that repression of respiratory activity did not play a crucial role in C. albicans morphogenesis. Although they have recently reported the preparation of petite mutants, they apparently did not attempt to see if these mutants were capable of forming germ tubes (Aoki and Ito-Kuwa, 1987).

Shepherd, Sullivan and co-workers have addressed themselves to the metabolism of N-acetylglucosamine (GlcNAc), with the idea of trying to show that the activities of the enzymes leading to the synthesis of chitin are enhanced during germ tube formation. However, once the GlcNAc is phosphorylated to GlcNAc-6-phosphate it can also be catabolized to fructose-6-phosphate. Their initial study (Shepherd et al., 1980b) showed that GlcNAc kinase was not a control point in dimorphism, there being no differences in its activity between the two phases, and inhibitors of germ tube formation having no effect on the enzymes's induction. They then compared the activities of the anabolic enzyme UDP-GlcNAc-pyrophosphorylase, with those of the catabolic enzymes GlcNAc-6-phosphate deacetylase and glucosamine-6-phosphate deaminase (Gopal et al., 1982). The activities of the catabolic enzymes increased 25- to 40-fold, while the anabolic enzyme increased 4-fold in the presence of GlcNAc. But these increases occurred in both germ tube forming and yeast growing conditions. The glucose analog 2-deoxyglucose, which inhibited germ tube formation but not yeast growth, inhibited the catabolic enzymes 40-60%, with no reported effect on the anabolic enzyme. Although they concluded that the increase in the anabolic enzyme may be significant, in accounting for the increase in GlcNAc incorporation during germ tube formation, the results were clearly not what they expected. Natarajan et al. (1984) reported similar results while studying the catabolic enzymes and concluded that the catabolism of GlcNAc and germ tube formation were mutually exclusive events. More recent evidence has demonstrated that a mutant unable to express the first three steps in the GlcNAc catabolic pathway (permease, kinase, and deacetylase) was incapable of being induced to form germ tubes with GlcNAc, but did form germ tubes with serum (Corner, et al, 1986). This would indi-

cate the metabolism of GlcNAc is required for germ tube formation to occur if GlcNAc is the only carbon source. However, the authors did not attempt to see if GlcNAc would induce the mutant to form germ tubes in the presence of glucose.

7. Differences in Other Subcellular Components

Differences were also found in other factors, such as cAMP (Bhattacharya and Datta, 1977; Niimi et al., 1980; Chattaway et al, 1981), DNA and RNA content (Yamaguchi, 1974; Wain, 1976; Schwartz and Larsh, 1980; Shepherd et al., 1980a; Anderson and Soll, 1984), actin localization (Anderson and Soll, 1986), polyphosphates (Cassone et al, 1983), and lytic enzymes (Ram et al., 1984, Sullivan et al., 1984; Molina et al, 1987). Their relationship to dimorphism have yet to be clarified.

B. Environmental Factors Affecting Germ Tube Formation

Although dimorphic fungi can be categorized according to the morphologies of their pathogenic and saprophytic states (Szaniszlo et al., 1983), one of the first attempts to categorize the dimorphic fungi was by the environmental factors which caused the transformation in vitro (Romano, 1965). In this system Blastomyces and Paracoccidiodes are examples of fungi which require only a change in temperature from 25°C to 37°C to bring about hyphal to yeast transformation (thermal dimorphism; Nickerson, 1948). The second group of dimorphic fungi, which includes Histoplasma and Sporothrix, consists of those organisms requiring both temperature and nutritional factors. Candida was grouped with Mucor in the third, and most complex group, in which nutrition or environmental factors alone or together induce morphoge-

nesis (Rippon, 1980). Although, this neat categorization is probably an oversimplification, as can be seen by the exceptions (Jacobson and Harrell, 1982; Rodriguez-del Valle et al., 1983; Kane, 1984), it clearly shows that many factors have been known for a long time to affect the dimorphism of C. albicans. This is most likely due to its popularity as a model organism for the study of dimorphism. On the other hand, it does not address itself to any interrelationships between these factors. In fact, until recently, most groups studying C. albicans were content to use whatever system they felt most comfortable with when studying dimorphism and tried to explain this phenomenon without ever attempting to see if their hypotheses applied when the cells were induced using other systems (Sevilla and Odds, 1986b). Unfortunately, attempting to look for a common factor or trying to understand why so many factors affect germ tube formation is complicated by the fact that the results of so many groups are contradictory. Table 1 lists a number of the nutrients and some the accompanying physical conditions used to obtain germ tubes. This is not meant to be an all inclusive table, but should give an indication of the multitude of conditions which have been used in the past. Other tables have been published in Odds (1979, 1985), and Hazen and Cutler (1983).

The goal of studying different types of inducers of dimorphism was originally to determine which metabolic pathway, or metabolic state, was most conducive to germ tube formation. Nickerson and associates' original hypothesis concerning dimorphism was based on the effects of cysteine and other reducing agents on germ tube formation (Nickerson, 1948; Nickerson and van Rij, 1949). Land et al, (1975a, 1975b) thought that since the most active inducers, proline, arginine and alanine, were all metabolized through glutamate

Table 1. Media used for induction of germ tubes in C. albicans

No:	C source	N source	Other media or conditions	Solid/ Liquid	pH
COMPLEX MEDIA					
1.	starch, 0.2%	neopeptone, 5%	yeast extract, 0.1%	L[1]	---[2]
2.	starch (reducing sugar free), 2%	$(NH_4)_2SO_4$, 1%	biotin, KH_2PO_4, 1%	S[3]	---
3.	soluble starch, 1%	tryptone 1%, casamino acids, 1%	CO_2, 10%	S	6.3
4.	---	trypticase soy broth, 3%	---	L	---
5.	zein, 4%	---	---	S	7.3-7.6
6.	corn meal, 4.17% (w/v)	---	coverslip	S	6-7
7.	yellow corn meal, 4%	---	---	S	---
8.	corn meal, 4.17%	---	reduced O_2, coverslip	S	6.2
9.	carrot infusion	---	---	S	---
10.	cream of rice, 1%	oxgall, 1%	Tween 80, 0.1%, coverslip	S	---
11.	---	serum (human or sheep)	---	L	---

Inoculum	Temp. °C	Time	Hyphal formation	Comments	Ref.
---	40	18 h	mostly hyphae		57
heavy streak	25	4 d	some hyphae	some yeast, many chlamydospores. glucose or cysteine resulted in yeast form.	209
thin streak	37 (25)	16-48 h	all 22 strains	some strains formed more hyphae at 25°C than at 37°C	141
< 5 X 10^5 cells/ml	37	3 h	80-95%, 79 strains		147
streak & cut agar	25	24-48 h	some hyphae	chlamydospores, pseudohyphae; not as effective as corn meal.	241
cut agar	25	2-3 d	some hyphae	pseudohyphae, chlamydospores	27
---	25	24 h	some filaments	chlamydospores	30
---	30-37, 22-26	6 d		curving hyphae, no chlamydospores at 37°C, some true hyphae at 24°C.	337
----	37.5	24 h	mostly hyphae	some pseudomycelia	80
light streak	37	3 h	10-90% in 100 strains	transfer to 25°C resulted in chlamydospore production by most strains.	24
loopful	37	3 h	95% of 86 strains		78

Table 1. (cont.)

No.	C source	N source	Other media or conditions	Solid/ Liquid	pH
12.	--	serum (many)	---	L	---
13.	---	human plasma, serum, or blood; egg or oleic acid albumin,	---	L	---
14.	---	human serum	---	L	---
15.	---	egg white	---	L	---
16.	glucose, 0.1%	glycine, 0.1%	yeast extract, 0.01%	L	6.8
17.	glucose, 0.1%	glycine, 1%	yeast extract, 0.1%, $NaHCO_3$	L	7.5
18.	---	---	saliva diluted 5:6 with 0.9% NaCl	L	---
19.	---	---	sandy soil (loam)	S	---
DEFINED MEDIA					
20.	---	eosin methylene blue	CO_2, 10%	S	---
21.	glucose, 0.5%	asparagine, 0.1%, (BSA 0.5%)	salts, Tween 80, 0.05%	L	7.0-7.3

Inoculum	Temp. °C	Time	Hyphal formation	Comments	Ref.
$<10^7$ cells/ml	37	3 h	100%	$>10^5$ cells/ml length decreased, $>10^7$ cells/ml inhibited hyphal formation.	177
---	37?	3 h	true hyphae	amino acids did not induce hyphal formation.	244
---	37-42	1.5 h	mostly hyphae		318
---	30-42	30-90 min	mostly hyphae		43
---	28	24 h	---	pseudohyphae (?)	207
10^6 cells/ml	37	4 h	70%		203
10^9 cells/ml (?) (McFarland scale)	37	18 h	10-300 g.t./ field in 13/25 samples	only saliva from ill patients yielded germ tubes.	15
---	25	3 d	some hyphae	chlamydospores, pseudohyphae	11
---	37	24 h	hyphae and yeasts		336
1.5 X 10^6 cells/ml	37	3 h	75-90%	% germ tube dependent on inoculum age & size, albumin conc.; hyphae form in absence of albumin	29

Table 1. (cont.)

No.	C source	N source	Other media or conditions	Solid/ liquid	pH
22.	sucrose, 1%	asparagine, 0.2%, NH_4-citrate, 0.6%	salts, vitamins, biotin	S	5.0
23.	glucose, 1%	$NaNO_3$, 0.2% or amino acids, 1% or serum	salts	L	7.5(i) 6.0(f)
24.	glucose, 0.9%	Auto Pow MEM, glycine, 0.1%	PO_4^{-3}, 10 mM, salts, biotin	L	6.0
25.	glucose, 0.1%	---	coverslip	S	--
26.	GlcNAc, 2.5 mM	GlcNAc, 2.5 mM	$MnCl_2$, 0.1mM, imidazole buffer, 0.01 mM	L	6.6
27.	maltose or sucrose, 42 mM	$(NH_4)_2SO_4$, 3.7 mM	salts, biotin	L	5.2
28.	glucose, 10 mM	NH_4Cl, 10 mM	salts, biotin	L	---
29.	glucose, 70 mM	NH_4Cl, 25 mM	salts, biotin	L	7.0
30.	dextrose, 139 mM	L-α-amino butyric acid, 0.1%	salts, biotin	L	5.7-7.0
31.	glucose, 5 mM	tissue culture medium 199	salts	L	6.0-7.2
32.	glucose, 70 mM	8 amino acids, including Pro (4.3 mM), $(NH_4)_2SO_4$, 39 mM	salts, biotin	L	6.8
33.	---	Proline, 10 mM	KH_2PO_4, biotin	L	7.0

Inoculum	Temp. °C	Time	Hyphal formation	Comments	Ref.
---	24	2 d	many filaments	galactose yielded most hyphae, PO_4^{-3} inhibited hyphae.	192
20% turbidity at 620 nm	37	24-96 h	M > Y		145
10^6 cells/ml	40	3-72 h	100%		266
---	37	3 h	97% of 257 strains	used only fresh colonies	49
8 X 10^5 cells/ml	37	5 h	> 90%		286
2.5 X 10^8 cells/L	28	24 h	hyphae, pseudo-hyphae		280
10^6 cells/ml	37	6 h	hyphae	lower glucose - no hyphae	161
10^7 cells/ml	37	6 h	0-100%, strain dependent		180
6 X 10^6 cells/ml	37	3 h	44-97%		185
1 X 10^6 cells/ml	37	3 h	97%		72
25% T at 550 nm	37	2-27 h	90%	no hyphae on solid media	164
1 X 10^6 cells/ml	37	3 h	86%	glucose, NH_4^+, and PO_4^{-3} depressed hyphal formation	72

Table 1. (cont.)

No.	C source	N source	Other media or conditions	Solid/ liquid	pH
34.	glucose, 10 mM	proline, 10 mM alanine, arginine, histidine, or isoleucine,	salts, biotin	L	---
35.	---	glutamate, 150 mM	salts, biotin	L	6.0
36.	glucose, 2.5 mM	glutamine, 2.5 mM	$MnCl_2$	L	6.6
37.	glucose, 2.5 mM	ManNAc, 2.5 mM	$MnCl_2$	L	6.6
38.	---	---	dH_2O[4]	L	---

1. Liquid media
2. Data not indicated
3. Solid media
4. Distilled water

Inoculum	Temp. °C	Time	Hyphal formation	Comments	Ref.
1 X10^6 cells/ml	37	6 h	93% (Pro), 10-90% (others)	PO_4^{-3} inhibitory	161
5 X 10^5 cells/ml	34-43	16 h	100%	glucose and cysteine were not inhibitory	214
8 X 10^7 cells/ml	37	4 h	90%		277
8 X 10^7 cells/ml	37	4 h	50%	ManNAc not metabolized	309
?	37	3 h	30% in 6.4% of isolates		149

and α-ketoglutarate, that the resultant synthesis of NADH would be at the expense of NADPH generation, which according to Nickerson's model was required for cell division (Nickerson, 1954, Dabrowa and Howard, 1983). The most interesting proposal, as mentioned in the beginning of this chapter, was that the inducers did not effect dimorphic change through their metabolism, but rather through an interaction with the cell membrane (Sullivan and Shepherd, 1982). In the interest of not being redundant, since much of this material will be mentioned in subsequent chapters, the relevant information will be discussed in detail in the appropriate chapters.

The initial intent of this project was twofold. One was to search for some determinant, by which it would be possible to effectively differentiate between germ-tube-forming and budding yeast cells before the germ tubes themselves were actually visible. Second, was to determine if the formation of germ tubes required the metabolism of the inducers, or whether the inducers acted as external stimuli. It was assumed that the basic conditions required for germ tube formation were adequately known. However, it became clear very early in the course of research that this was not the case. What was generally accepted as optimal conditions for germ tube formation were found to be affected by even the simple components of the induction solution. Furthermore, the presence of even minute amounts of contaminants in the induction solution affected the results. The major emphasis of the project then turned to identifying those factors which were considered essential to germ tube formation.

The first chapters will describe the preliminary results which led the research in the direction it ultimately took, followed by the findings

on the interrelationship between pH, temperature, glucose and bicarbonate concentration. The final experiments led to the conclusion that carbon dioxide and/or bicarbonate concentration are the ultimate determinants of the dimorphic transition of C. albicans. While I did not determine conclusively that it was the metabolism of CO_2 itself which leads to the yeast-hyphal transition, the final discussion in this dissertation will argue that it is the most plausible explanation for this phenomenon.

CHAPTER II

PRELIMINARY EXPERIMENTS

INTRODUCTION

The accepted hypothesis at the time that this research was initiated, was that the cell wall, and in particular the percent of chitin in the wall, was implicated in the dimorphism of C. albicans. Since the studies which supported this view used only one type of medium to grow the cells, we thought it might be useful to compare the cell wall compositions of both the hyphal and yeast forms after growth on different media. In addition, we were interested in determining what other compounds, especially whether analogs of known inducers, such as GlcNAc and proline, could also induce germ tube formation. The possible induction by analogs of these inducers would support the thesis that the metabolism of these inducers was not required for germ tube formation to occur.

It became clear very early, that germ tube formation could occur under ostensibly endotrophic conditions, that is in the absence of either exogenous metabolizable nitrogen or organic carbon sources. Although, there are previous reports of limited germ tube formation in water and on agar without added nutrients (Joshi et al., 1973, 1975), endotrophic germ tube formation had never been systematically studied. If this finding could be verified, it would clearly prove that the metabolism of "inducers" was not required for germ tube formation. Just as important, it would serve as a catalyst in understanding the underlying mechanism(s) of germ tube formation.

It was imperative that this issue be resolved early, since the unexpected appearance of germ tubes in what were to serve as negative (yeast-forming) control experiments could not be tolerated. It was later determined that minute amounts of ethanol, present as a contaminant in the buffers used, were responsible for what was originally thought to be endotrophic germ tube formation. The evidence supporting this conclusion will be presented in the first part of this chapter. We were able to demonstrate at the end of this research, though, the actual existence of nominally endotrophic germ tube formation induced by carbon dioxide and/or bicarbonate. This supporting evidence will be presented in Chapter Seven. The second part of this chapter will deal with some general factors affecting germ tube formation. The final part of this chapter will present the preliminary experiments on the cell wall composition, which led us to believe that we would not find an explanation for germ tube formation by studying that area.

MATERIAL AND METHODS

Organism. C. albicans, ATCC 58716 was routinely used for the experiments described in this paper. Cells were maintained on Sabouraud dextrose agar (SDA, Difco Laboratories) at 20°C.

Growth and preparation of organism. Yeast phase cells were prepared by growing the cells on SDA for 24 h at 37°C. The cells were collected and washed with glass distilled, deionized water ten times by vacuum filtration on a Millipore filtration apparatus, with the cells being resuspended in water by repeated suction and forcible ejection through a pasteur pipette before each washing. In some experiments the cells were starved by incubation in water at 20°C for 16 to 24 h. These cells were referred to as preconditioned or primed cells.

Induction of germ tube formation. Washed C. albicans yeast-phase cells (final concentration of 8 x 10^5 cells ml^{-1}, except when indicated otherwise) were inoculated into a buffered solution (final volume, 0.5 ml) containing the following salts: $FeSO_4$ (0.1 mg L^{-1}), KCl (400 mg L^{-1}), $MgSO_4.H_2O$ (200 mg L^{-1}), $NaH_2PO_4.H_2O$ (125 mg L^{-1}), NaCl (3200 mg L^{-1}), and $NaHCO_3$ (750 mg L^{-1}). The buffers used were ACES (N-[2-acetamido]-2-aminoethanesulfonic acid), HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), MOPS (3-[N-morpholino]propanesulfonic acid), PIPES (1,4-piperazinediethanesulfonic acid), sodium phosphate, Tris(tris[hydroyxymethyl]amino methane)-hydrochloride and Tris-maleate. All buffers were adjusted to the desired pH (7.2 unless indicated otherwise) with NaOH. The concentration of the buffer salt was

0.05 M. In those experiments utilizing ethanol, ethanol was added to the above solution to obtain a final concentration of between 17 μM and 17.1 mM (0.0001% to 0.1% v/v). In some experiments germ tubes were induced by adding either proline, GlcNAc (each 4 mM) or the combination of glucose and NH_4Cl (each at 5 mM) to the above solution. In some control experiments germ tubes were induced with Dulbecco's modified eagle medium, (DMEM, Gibco Laboratories) or with a solution of 0.1% Casitone (Difco). The cells were incubated at 37°C for 4 h, shaking the tubes manually, at 30 min intervals. The percentage of cells forming germ tubes was measured by counting 100 to 200 cells (or cell clusters) in each sample with a phase contrast microscope (Nikon). Although, generally germ tubes were formed from single cells, in those instances when they were formed from budded cells they were counted as one "germ tube unit" regardless of whether both the mother and daughter or only one of them had a germ tube.

Gas chromatographic analysis for the presence of ethanol. Aqueous solutions of the buffers (0.2 M) were analyzed for the presence of ethanol by gas chromatography. Aliquots (2 μl) were injected into a Perkin-Elmer (model 910) gas chromatograph equipped with a 183 cm by 2 mm (inner diameter) 60/80 Carbopack B-5% Carbowax 20m column (1-1766 Supelco). The injection temperature was 190°C, the column temperature was 110°C, and the flame ionization temperature, 210°C. The flow rate was 20 ml min^{-1} of helium. The signal to noise ratio at the lowest level of ethanol detected was approximately 7:1.

Preparation of Cell Walls. Both yeast and hyphal walls were prepared by

breakage with glass beads using the procedure of Van Etten and Freer (1978). Approximately 1-2 g (wet weight) of washed cells, suspended in 3 ml ice cold distilled water, were vortexed with 10 g acid-washed glass beads (0.5 - 1 mm, Sigma Chemical Co.) in a parafilm-sealed 30 ml Corex centrifuge tube (Corning Glass). Five to eight 1 min treatments were sufficient to achieve greater than 95% breakage of the yeast cells, but the hyphal cells required at least 20 min vortexing to achieve similar results. Each treatment was followed by a brief wash by centrifugation (1,500 x g, 3 min) in cold distilled water to remove cytoplasmic debris and to minimize cell wall degradation by endogenous enzymes.

The cells were washed 10 times in distilled water by centrifugation (1,500 x g, 5 min), with mild sonic oscillation with a standard tip (position 2, [=2 A], model S-75 sonifier, Branson) for 30 s used between washings to remove adhering cytoplasmic particles and membranes. This was a little difficult in the case of the hyphal walls as they tended to clump and adhere to the glass in cold water, and metal particles from the sonifier tip also tended to adhere to all but the ethanol-induced hyphal walls. The final washes were then done using water warmed to room temperature. The walls were checked by phase contrast microscopy for the absence of cytoplasmic contamination, and then lyophilized and stored under a vacuum until use.

Chemical Analysis of Cell Walls. To analyze cells for total neutral sugars, the anthrone procedure was utilized (Morris, 1948). To determine total protein the cell walls were first extracted with freshly prepared 1 N NaOH in a boiling water bath for 1 h. The walls were centrifuged at 1,500 x g, the supernatant was removed and the residue was extracted once more. The walls

were then centrifuged again and the supernatant which was combined with the previous supernatant was analyzed using the method of Lowry et al. (1951). Bovine serum albumin (crystallized and lyophilized, Sigma) was used as the standard. The NaOH-extracted walls were then washed until the supernatant was neutral and then lyophilized and dried. The alkali-extracted residues were then hydrolyzed with 2 ml 6 N HCl in sealed glass ampoules for 4 h in a boiling water bath (Blumenthal and Roseman, 1957). The hydrolysates were neutralized with 6 N NaOH and the volume brought up to 8 ml, so that the final concentration of NaCl was 1.5 M. Hexosamines were then quantitated using a modified Elson-Morgan test (Winzler, 1955), with all control samples of glucosamine.HCl being diluted in 1.5 M NaCl. Detailed descriptions of these procedures may be found in Pollack (Master's thesis, Loyola University of Chicago, 1982).

Chemicals. Except where otherwise indicated, all sugars were the D-anomer and all amino acids were the L-anomer. Most of the sugars used in this study were obtained from Sigma Chemical Co. The exceptions were N-acetyl-glucosamine, glucosamine (Pfanstiel), and glucose (Mallinckrodt). All chemicals were used without further purification, except as indicated in the case of the buffers.

RESULTS

Endotrophic and ethanol-induced germ tube formation

The tris-maleate buffer system was originally used in this laboratory due to the large pH range that was attainable with it, and also because it apparently reduced clumping of germ tube forming cells induced by DMEM, a very effective inducer of germ tubes (W. Tatarowicz, Master's Thesis, Loyola University of Chicago, 1984). The salts used were those found in DMEM. Although in the one report of germ tube formation in water, up to 30% of the cells of a few strains were capable of forming germ tubes (Joshi et al., 1973), there was no precedent for any buffer or salt being a stimulator of germ tube formation. Tatarowicz (Master's Thesis, 1984) had shown that priming of the cells enabled cells induced with arginine or glutamine to form germ tubes quicker, and under certain instances, for a greater percentage of cells to form germ tubes. Therefore, we initially used exclusively primed cells. Frequently, although not always, between 40 and 70% of the cells formed short (< 10 μm long) germ tubes in the buffered salts solution, without the addition of any nutrient. Less than 20% of non-primed cells, however, formed germ tubes, in the same solution. Similar values were obtained using HEPES or ACES buffers. Although the evidence seemed to indicate that germ tube formation was endotrophic, there were a number of buffers in which germ tube formation did not occur. These included sodium phosphate, Tris-HCl, MOPS, PIPES and even Tris-maleate when prepared from each separate buffer component instead of the premixed commercial buffer, or from a commercial preparation with a different lot number. Furthermore, when the solubilized commercial buffers were first lyophilized thoroughly and then

rehydrated before use, the primed cells no longer formed germ tubes in the buffered salts solution (Table 2). After learning that ethanol was used in the preparation of the commercial buffers, we determined that final concentrations of ethanol as low 0.0001% could induce germ tube formation. Gas chromatographic analysis of buffers capable of supporting germ tube formation revealed them to contain between 0.0002 and 0.004% (v/v) ethanol in a 0.05 M solution (Table 2, Fig. 1).

The effect of priming and priming temperature on ethanol-induced germ tube formation

Since priming of the cells was required to visualize the "pseudoendotrophic" germ tube formation, we wished to further characterize some of its effects. The first effect was enabling extremely low concentrations of ethanol to induce germ tube formation. In the absence of priming, concentrations of ethanol below 0.01% were significantly less able to induce germ tube formation relative to cells that were primed (Fig. 2, Fig. 3). This effect was also more evident when the cells were induced at pH 7.2 rather than when they were induced at pH 5.8, the optimal pH for ethanol-induced germ tube formation (See Chap. 3). This is apparently the reason why "pseudoendotrophic" germ tube formation was not observed using non-primed cells. The second effect characterized was the effect of the priming temperature. The optimal temperature was found to be 20°C. Incubation in water at 30 to 37°C for 24 h, however, resulted in almost complete inhibition of ethanol-induced germ tube formation, and approximately 50% inhibition of GlcNAc- or proline-induced germ tube formation. DMEM-induced germ tube formation, however, was hardly affected. However, the addition of 2 to 8 mg/ml sodium

Table 2. Evidence that ethanol present in commercial buffer salts is responsible for triggering germ tube formation in C. albicans.[1]

Buffer salts used in incubation solution	% of cells forming germ tubes	Concentration of ethanol contained in 0.05 M buffer (% v/v) (mM)
Tris-maleate (Sigma 109C-5022)	66	0.0005 (0.09)
Tris-maleate (Sigma 28C-5052)	75	0.001 (0.17)
HEPES (Sigma 80F- 5059)	73	0.004 (0.68)
ACES (Sigma 73C-0810)	71	0.0002 (0.03)
Tris-maleate (Sigma 61C-5130)	9	ND[2]
Tris-maleate (made from Trizma base [Sigma 104C-5000] and maleic acid [Sigma 126B-5490] components)	8	ND
Tris-maleate (Sigma 109C-5022) (solubilized and lyophilized) plus:		
0% Ethanol	4	ND
0.001% Ethanol	68	0.001 (0.17)
0.01% Ethanol	66	0.01 (1.71)
0.1% Ethanol	66	0.1 (17.1)
DMEM (control)	100	ND

1 The values for the % cells forming germ tubes are the means of at least 4 independent experiments. This table is from J. H. Pollack and T. Hashimoto, 1984, Appl. Environ. Microbiol. 48: 1051-1052.

2 Not determined

Figure 1. Determination of ethanol in commercial Tris-maleate by gas chromatography. The results were obtained by injecting 2 μl samples into a Perkin-Elmer gas chromatograph as described in Material and Methods. The concentration of ethanol in B and C was 0.002% (0.34 mM). (This figure is from J. H. Pollack and T. Hashimoto, 1984, Appl. Environ. Microbiol. 48: 1051-1052.)

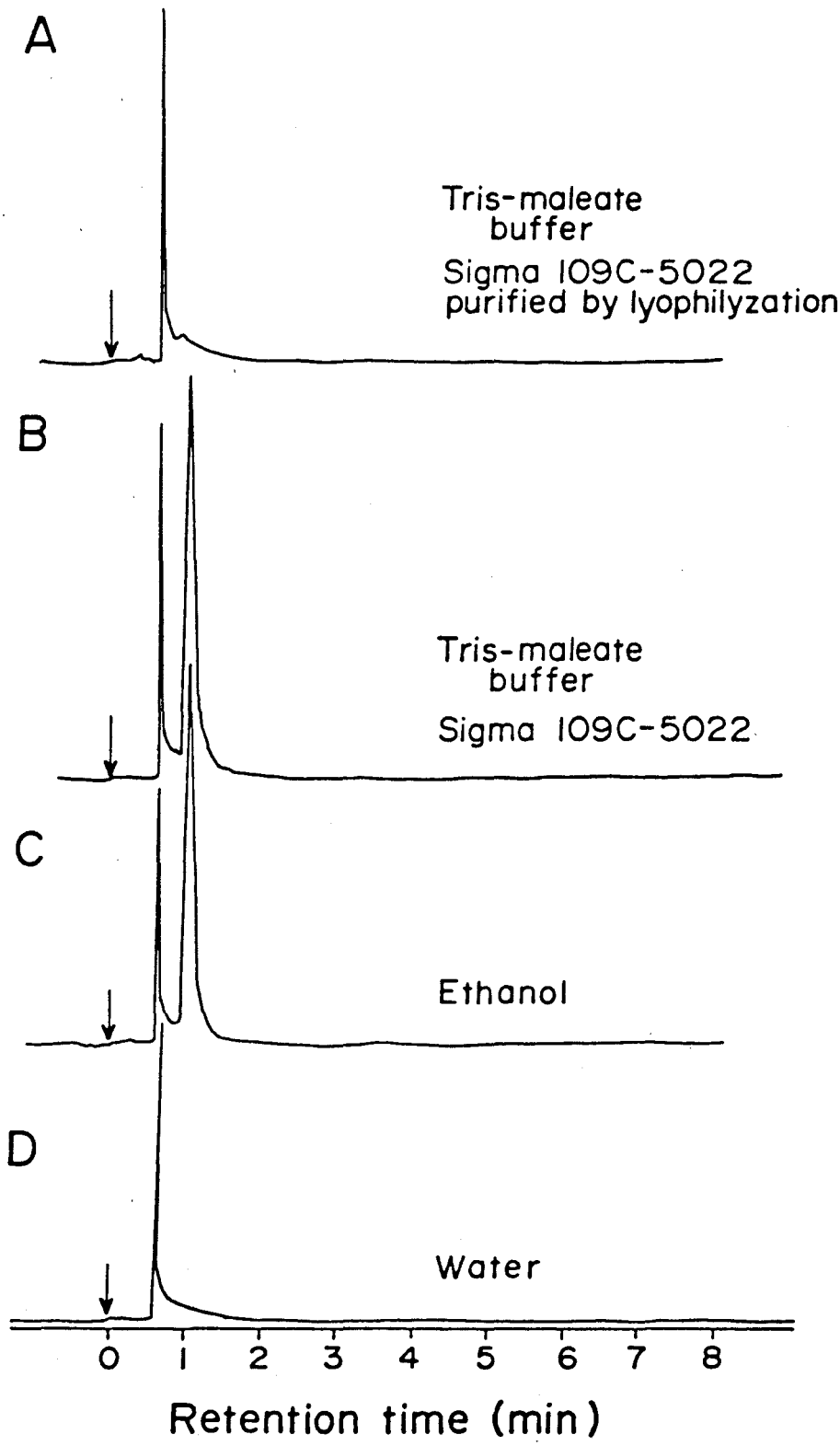

A
Tris-maleate
buffer
Sigma 109C-5022
purified by lyophilyzation
B
Tris-maleate
buffer
Sigma 109C-5022
C
Ethanol
D
Water
0
1
2
3
4
5
6
7
8
Retention time (min)

Figure 2. Effect of priming temperature on germ tube formation. Cells were preincubated in water at the indicated temperatures for 24 h. They were then induced to form germ tubes with different concentrations of ethanol at pH 5.8, and incubated for 4 h at 37°C. Values are the means of 3 independent experiments. Error bars indicate SEM. Solid bar, 0.1% ethanol; Clear bar, 0.01% ethanol; Striped bar, 0.001% ethanol. Control, cells not preincubated.

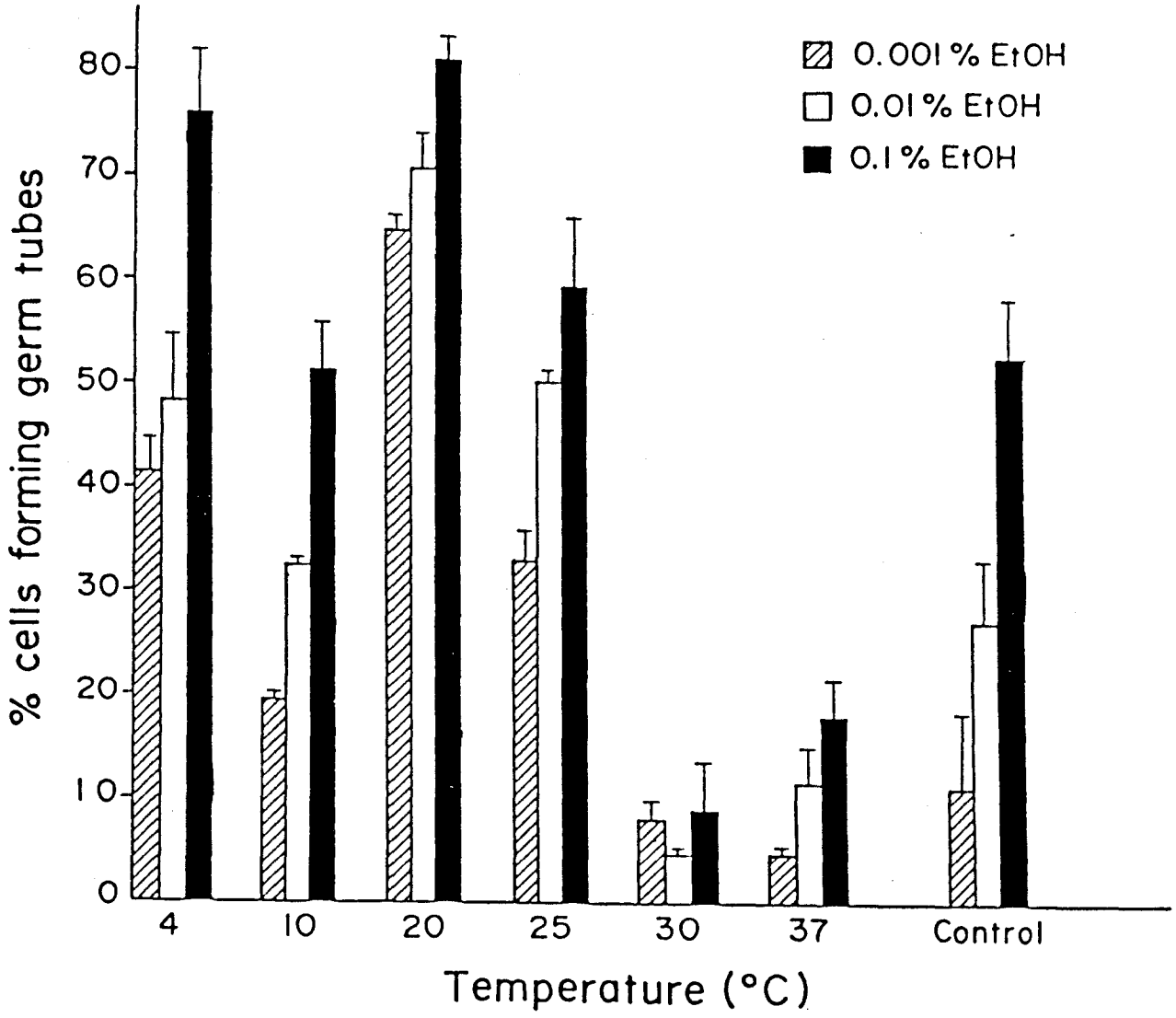
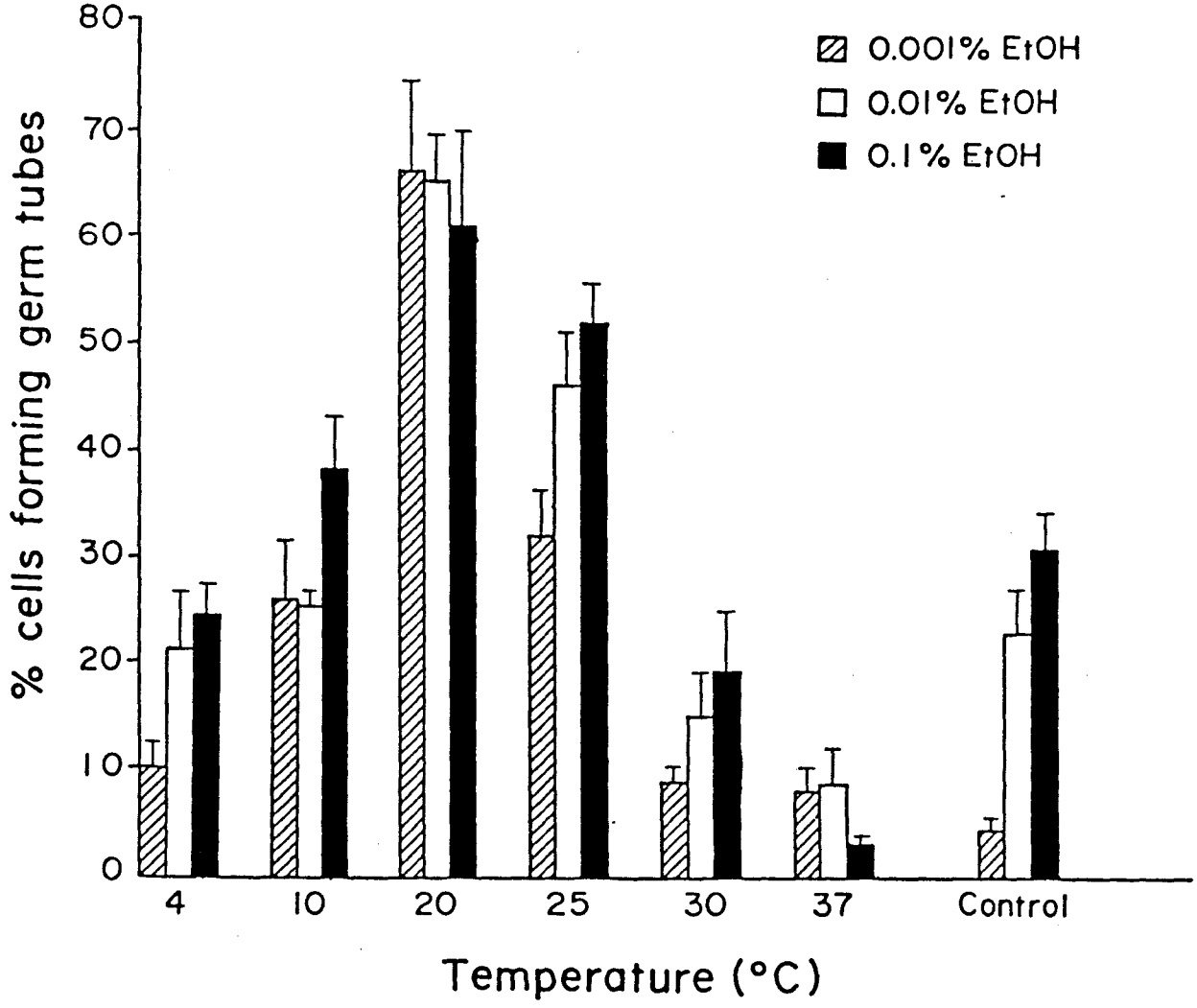

0.001 % EtOH
0.01 % EtOH
0.1 % EtOH
% cells forming germ tubes
0
10
20
30
40
50
60
70
80
4
10
20
25
30
37
Control
Temperature (°C)

Figure 3. Effect of priming temperature on germ tube formation. Cells were preincubated in water at the indicated temperatures for 24 h. They were then induced to form germ tubes with different concentration of ethanol at pH 7.2, and incubated for 4 h at 37°C. Values are the means of 3 independent experiments. Error bars indicate SEM. Solid bar, 0.1% ethanol; Clear bar, 0.01% ethanol; Striped bar, 0.001% ethanol. Control, cells not preincubated.


0.001% EtOH
0.01% EtOH
0.1% EtOH
% cells forming germ tubes
80
70
60
50
40
30
20
10
0
4
10
20
25
30
37
Control
Temperature (°C)

bicarbonate to the final incubation solution completely overcame this suppression (data not shown).

Effect of cell concentration on germ tube formation

Since ethanol-induced germ tube formation was greatly affected by the cell concentration (Chap. 3), the effect of cell concentration was tested in the DMEM-, proline- and GlcNAc-induced systems. No deleterious effects were noted in any of these systems at concentrations up to 3 X 10^6 cells ml^{-1}. Above that concentration, there was a gradual decrease in the percentage of cells forming germ tubes in the proline-induced systems until only approximately 20% of the cells formed germ tubes at a concentration of 1.5 X 10^7 cells ml^{-1}. This value could be partially ameliorated by increasing the concentration of proline. A decrease in the DMEM-induced system was noticed at 9 X 10^6 cells ml^{-2}. A decrease in the percentage of cells forming germ tubes in the GlcNAc-induced system was only detected at the highest concentration of cells tested (1.5 X 10^7 cells ml^{-1}). Increasing the concentration of GlcNAc could also relieve this inhibition of germ tube formation (Table 3).

Effect of salt concentration on germ tube formation

The concentration of NaCl in DMEM is approximately 219 mM. However, since DMEM was used at a dilution of 1:1 for induction experiments, the cells were exposed to only half that concentration of NaCl. We wished to ascertain that such a high concentration would not be inhibitory to the induction systems we were using. Significant reduction in the percentage of cells forming germ tubes occurred at a concentration of 100 mM NaCl in the

Table 3. Effect of cell concentration on germ tube formation.[1]

Cell concentration (Cells X 10^6 ml^{-1})	DMEM-induced cells	Proline-induced cells	GlcNAc-induced cells
1.5	100	94	100
3	98	ND[2]	95
4.5	ND	82	93
6	ND	71	87
7.5	97	74	97
9	77	66	89
12	ND	48	93
15	ND	22(35)[3]	71(97)[4]

1 Cells were induced in buffered salts containing 4 mM GlcNAc or proline, or with DMEM diluted 1:1. Values are the means of 2 experiments.

2 Not determined

3 Value in parenthesis was obtained when cells were induced with 400 mM proline.

4 Value in parenthesis was obtained when cells were induced with 80 mM GlcNAc.

proline-induced system. The concentration of NaCl in our salt solution was, therefore, subsequently lowered to 50 mM. Although a systematic study was not made for other salts, a number of other salts were found to inhibit germ tube formation at similar concentrations (Table 4).

Survey of compounds which induce or inhibit germ tube formation

Having identified conditions which did not result in endotrophic germ tube formation, we were then able to identify compounds which induce or inhibit germ tube formation. Significant germ tube formation did not occur in the presence of most amino acids, citric acid cycle intermediates, or amino sugars. However, as has been well reported, certain amino acids, such as γ-amino butyric acid, arginine, glutamic acid, glutamine, and proline are fairly good inducers of germ tube formation. Of the amino sugars tested, GlcNAc is an excellent inducer, although glucosamine can also induce about 65% of the cells to form germ tubes. However, of the analogs of proline tested, only the three esters were capable of inducing germ tube formation. None of the analogs of GlcNAc, with the exception of glucosamine, demonstrated significant inductive potential. The addition of 5 mM of glucose to some poor inducers, such as δ-amino valeric acid, prolinamide, β-alanine, and α-amino butyric acid, significantly increased germ tube formation (Table 5).

A number of compounds, especially those which are thought to have effects on the cell wall, or cell wall enzymes, were also tested for their ability to inhibit germ tube formation. None of the compounds tested were inhibitory at concentrations reported to inhibit wall enzymes. However, inhibition did occur in some instances at very high concentrations, though (Table 6). Removal of the outer layer or the cell wall with DTT/EDTA/pronase

Table 4. Inhibition of germ tube formation by salts.[1]

Salt	Concentration (mM)	Percentage of cells forming germ tubes
NaCl	0	95
	50	88
	100	69
	200	65
	350	37
	500	5
$CaCl_2$	100	56
KCl	100	100
	1000	0
KH_2PO_4	50	87
	500	24
$MgSO_4$	100	73
	1000	15
$MnSO_4$	100	73

1 Cells tested for NaCl effect were induced by proline (4 mM) while those tested for the other salts were induced by GlcNAc (4 mM). Values are the means of two experiments.

Table 5. Induction of germ tubes by amino sugars, amino acids and their analogs.

Compound (5 mM)	Percentage cells forming germ tubes
Poorly stimulatory amino acids and related compounds[1]	<10
Stimulatory amino acids	
alanine	61
γ-amino butyric acid	75
arginine	84
aspartic acid	37
glutamine	64
glutamic acid	85
tryptophan	22
Analogs or metabolites of proline	
poorly stimulatory analogs[2]	<15
proline benzyl ester	91
proline butyl ester	95
proline methyl ester	95
proline-agarose	0
Analogs of N-acetylglucosamine	
chitobiose	20
chitin	6
GlcNAc-agarose	2
N-acetylgalactosamine	17
N-acetylmannosamine	15
glucosamine	65
Glycolysis, citric acid cycle intermediates and related compounds[3]	<10
sodium acetate	31
Adenine	1
Adenosine	15
Malonic acid	5

1 The following amino acids were tested: β-alanine (47), N-acetyl-L-alanine, N-acetyl-L-glycine, N-acetyl-L-histidine, α-amino butyric acid (51), cysteine, histidine, homoserine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, serine, threonine, valine. The addition of 5 mM glucose to any of these compounds was without effect except where indicated by a value in parentheses. Values are the means of two experiments.

Table 5 (cont.)

2 The following compounds were tested: N-acetylproline, alanyl-proline, δ-amino valeric acid (57), dipicolinic acid, 2-furaldehyde, hydroxyproline, picolinic acid, pipecolic acid, prolinamide (96), prolyl-alanine, L-prolinol, pyrrole, pyrrole-2-carboxylic acid, pyrrolidine, quinolinic acid. The addition of 5 mM glucose to any of these compounds was without effect except where indicated by a value in parentheses.

3 The following compounds were tested: glucose, glycerol, L-malic acid, sodium citrate, sodium glyoxylate, sodium pyruvate, sodium succinate.

Table 6. Inhibitors of germ tube formation.

Compound	Concentration	Type of Inhibitor	% cells forming germ tubes
Disulfiram	0.5 μg ml^{-1}	aldehyde dehydrogenase	93
	1.0 μg ml^{-1}		55
	2.0 μg ml^{-1}		0
4-Iodopyrazole	0.75 mM	alcohol dehydrogenase	100
Glucono-γ-lactone	10 mM	glucosidase	100
Mannono-γ-lactone	10 mM	mannosidase (?)	100
p-Chloromercurophenyl sulfonic acid	20 μg ml^{-1}	mannosidase, sulfhydryl blocking agent	95
	100 μg ml^{-1}		57
	500 μg ml^{-1}		0
Cycloheximide	50 μg ml^{-1}	protein synthesis	99
EDTA	1 mM	mannosidase	99
$CuSO_4$	1 mM	mannosidase	94
Trypsin inhibitor	0.9 mg ml^{-1}	trypsin	98
$HgCl_2$	1 μM	glucanase	97
	2 μM		50
	20 μM		0
Thioglucose	4 μM	glucose analog	77
	40 μM	alcohol dehydrogenase	52
	400 μM		0
DMSO	5%	permeabilyzer	97
Polyoxin D	50 μg ml^{-1}	chitin synthetase	86
Nikkomycin X & Z	1 mg ml^{-1}	chitin synthetase	90
Nikkomycin +DMSO			72
Ketoconazole	10 mg ml^{-1}	growth	100
	50 mg ml^{-1}		62

1 Cells were induced with 5 mM GlcNAc dissolved in Tris-maleate buffered (pH 5.8) salts solution. Values are the means of two experiments.

(Torres-Bauza and Riggsby, 1980) also had no effect on induction of germ tubes by proline or GlcNAc (Table 7).

Chemical composition of cell walls

The cell walls of both the yeast and hyphal forms were analyzed for protein, neutral and amino sugars, for purposes of comparison. Approximately 50% of the both wall types consisted of neutral sugars, 10% protein, and 10% amino sugars. No significant differences were found between the yeast and hyphal walls, nor between the compositions of hyphal walls produced by different inducers (Table 8). In addition, digesting either wall type with glusulase (snail gut enzyme containing both chitinase and β(1->3)-glucanase activities, Endo Laboratories, Garden City, N. Y.) resulted in essentially complete digestion of the walls when viewed microscopically. Thus, neither wall type contained the chitinase-resistant fraction which distinguished the arthroconidia walls of Trichophyton mentagrophytes (Pollack et al., 1983). Although the totals do not add up to 100% of the wall weight, it was decided to discontinue this line of research since no major distinctions could be found between the two wall types.

Table 7. Effect of removal of wall components on germ tube formation.[1]

Agent	% cells forming germ tubes
NaOH (0.01 M)	64[2]
(0.05 M)	0
HCl (0.1 M)	89[2]
(0.2 M)	49
(0.5 M)	0
Mixed glycosidase	92
DTT/EDTA/Pronase (Sigma)[3]	85
DTT/EDTA/Pronase (Calbiochem)	63

1 Cells were exposed to each of the agents for 3 h, washed 10 times to remove any traces of the agents and then were induced with 5 mM GlcNAc for 4 h. Values are the means of two experiments.

2 Cells induced with ethanol (0.1%) did not form germ tubes.

3 DTT (40 mM), EDTA (5 mM), Pronase (1 mg ml^{-1}), Tris-HCl buffer, pH 8.5.

Table 8. Chemical composition of the yeast and hyphal walls of C. albicans.

Component	Composition (%)	
	Yeast wall	Hyphal wall
Neutral sugars[1]	55.2	47.0
Amino sugars[2]	8.0	
GlcNAc-induced		9.0
Ethanol-induced		7.6
Alkali residue	19.0	
DMEM-induced alkali residue		17.8
GlcNAc-induced alkali residue		23.4
Ethanol-induced alkali residue		14.9
Protein[3]	7.4	
DMEM-induced		10.2
GlcNAc-induced		10.4
Ethanol-induced		10.0

1 Values determined by the anthrone procedure. All values are the means of two experiments.

2 Values determined by Elson-Morgan procedure.

3 Values determined by Lowry procedure.

DISCUSSION

Most attempts to explain dimorphism have centered around cell wall changes or enzymes related to cell wall synthesis (see General Introduction). We also thought it probable that some relationship could be found between dimorphism of C. albicans and changes in the cell wall. However, in order to make sure that whatever differences were found were not related to the growth conditions, we induced hyphal growth by using three different media, ethanol, GlcNAc, and DMEM. Surprisingly, we did not find any significant differences in any of the three fractions studied, total sugars, amino sugars, and protein. Considering that 70% of the walls were extracted by alkali, the total chitin in the walls ranged from 4.5% in the ethanol-induced cells to 7.8% in the GlcNAc-induced cells. These values were not considered significantly different than the 5.9% found in the yeast walls. Although our values for the hyphal chitin were similar to those reported in the literature (Chattaway et al., 1968; Mattia et al., 1982), the values for yeast cells were considerably higher. In those reports of similar values of chitin in yeast cells (Schwarz and Larsh, 1980; Elorza et al., 1983a) the values of chitin in the hyphal walls was also correspondingly higher. As can be seen from the differences between the chitin values from the ethanol- and GlcNAc-induced cells, these differences may be media related. In any event, our results did not support a major change in the percentage of wall chitin, or other wall fraction, as a factor in dimorphism. It should be mentioned, though, that changes in linkages between the different polymers may still play a role in dimorphic change. Such a report of a unique linkage between chitin and β-(1→6) glucan, similar to those reported in Schizophyllum commune (Sonnenberg et al., 1985), has

recently been reported (M. Shepherd, Am. Soc. Microbiol. Conference on the Biology and Pathogenicity of Candida albicans (1987).

A number of attempts were made to see whether enzymes or chemicals which affect wall polymers also affect germ tube formation. Inhibitors of wall lytic enzymes seemed, for the most part, to be ineffective. This seems to be in agreement with previously published data (Ram et al., 1984; Molina et al., 1987), although wall lytic enzymes seem to be induced by GlcNAc. Even the apparent removal of the outer wall layer, consisting of the mannan-protein, seemed to be without effect. So it seems that there is no external wall receptor with which the inducers of germ tube formation react. In those instances where inhibitors did eliminate germ tube formation, it is most likely that they also were inhibiting other processes.

The most important result of this section was the discovery that minute amounts of ethanol were sufficient to induce germ tube formation in C. albicans. The particulars of ethanol-induction will be discussed in the next chapter. It was particularly interesting that ethanol-induced germ tube formation required priming of the cells. Priming was required only when small concentrations of ethanol, such as those found in some buffers, were used to induce germ tube formation. At the time of this observation, the role of priming was not very clear. It seemed that larger concentrations of ethanol (0.1%), especially at pH values lower than the neutral pH (5.8) previously considered optimal for germ tube formation, were possibly providing some nutrient which could also be accumulated through starvation. This could be ethanol itself. The starvation period could cause the cell to induce a "high affinity" uptake system (Harder and Dijkhuizen, 1983) which might allow low concentrations of ethanol to induce germ tube formation. However,

another explanation may be found from the observation that preincubation at 30 and 37°C is inhibitory to most germ tube formation. With the recognition of the role of bicarbonate (Chapter 7), in germ tube formation, I decided to add extra bicarbonate (4 mg/ml instead of 0.75 mg/ml) back to the solution following a 24 h preincubation at 37°C. Germ tube formation again reoccurred with all inducers including ethanol. Thus, starvation at 20°C may be causing the cells to produce and accumulate bicarbonate, which results in germ tube formation, even when small amounts of ethanol are added. At 37°C, when the solubility of CO_2 decreases, CO_2 may be lost to the atmosphere. Sims (1986) has suggested that CO_2 accumulation in vacuoles of C. albicans plays a role in morphogenesis. Whether vacuolation occurs in response to starvation, as it occurs during germ tube formation (Gow and Gooday, 1982, 1984), remains to be observed.

Carbon dioxide may also play a role in the decrease in the percentage of cells forming germ tubes as the concentration of cells in suspension increases. Dissolved CO_2, due to increased suspension densities, was thought to affect a number of metabolic processes in yeast (Janda and Kotyk, 1985). Although increasing the concentration of the inducer, which could provide more nutrients for CO_2 production, seemed to allow more cells to form germ tubes at higher concentrations, it did not appear to be a very significant increase. Alternatively, the decrease in nutrition caused by the increased density, may also suppress germ tube formation.

The lack of analogs of known inducers which were capable of inducing germ tube formation will be discussed in Chapter 6. The only analogs which seemed to be capable of inducing germ tube formation were proline esters. Since the cells most likely produced esterases, it is very likely that proline

itself would be available to the cell (Rossignol and Vary, 1979).

CHAPTER III

ETHANOL-INDUCED GERM TUBE FORMATION IN CANDIDA ALBICANS

ABSTRACT

Ethanol is the first reported compound which can induce germ tube formation in Candida albicans without the addition of any nitrogen-containing nutrients. Conditions controlling induction of germ tubes in C. albicans by ethanol were investigated. Ethanol (17.1 mM), in buffered salts solution containing sodium bicarbonate, induced 70-80% of yeast phase cells of C. albicans to form germ tubes. Germ tubes could be induced by ethanol (0.08 to 340 mM) at temperatures ranging from 29 to 41 °C (optimum 37°C) and at pH values ranging from 3.0 to 8.0 (optimum 5.8). The germ tubes averaged 11 μm in length after 6 h at 37°C. The percentage of cells forming germ tubes decreased as the concentration of cells in the induction solution was increased above 4 x 10^5 cells ml^{-1}. Germ tubes first appeared 45 to 60 min after continuous exposure to ethanol at 37°C and all cells which formed germ tubes did so by 2 h. Germ tube length decreased as pH was increased but was independent of the concentration of ethanol. Oxygen was required for germ tube formation. In addition to ethanol, 1-propanol, 2-propanol, 1-butanol and acetic acid could induce germ tube formation, whereas methanol could not. These results indicate that the cells must mobilize their endogenous nitrogen, and probably carbohydrate reserves, in order to initiate formation of

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This chapter is, in part, from J. H. Pollack and T. Hashimoto, 1985, J. Gen. Microbiol. 131: 3303-3310.

germ tubes. The evidence is inconclusive as to whether ethanol itself must be metabolized for germ tube induction to occur, although it is not thought to act by a nonspecific interaction with the cell membrane.

INTRODUCTION

Ethanol has been reported to induce a number of morphological changes in fungi. These changes range from the production of rhizomorphs, instead of mycelia, in Armillaria mellea (Weinhold, 1963), germination of macrospores of Fusarium solani (Cochrane et al., 1963; Paz et al., 1984), formation of Saccharomyces cerevisiae ascospores (Miller and Halpern, 1956), branching of germ tubes of Peronospora parasitica (McMeekin, 1981) and germ tube formation by Aureobasidium pullulans (Sevilla et al., 1983) and Candida tropicalis (Tani et al., 1979). There has been only one previous report of Candida albicans forming germ tubes when the growth medium was supplemented with ethanol (Reynouard et al., 1979). However, filamentation did not appear until the tenth day of exposure, at which time germ tubes were also reported in the control sample. Land et al. (1975) found that ethanol could not replace glucose in a proline/glucose germ tube induction medium. Chiew et al. (1982) reported that 4% ethanol inhibited germ tube formation, while Bell and Chaffin (1983) reported that growth of the yeast cells in medium containing even 0.1% ethanol as the carbon source, before induction using the medium of Lee et al. (1975), inhibited germ tube formation in C. albicans.

Previous findings that certain L-amino acids, such as proline, glutamine and arginine, or amino sugars, such as N-acetylglucosamine (GlcNAc) (Odds, 1979), or "gratuitous" inducers, such as N-acetylmannosamine or GlcNAc coupled to agarose (Sullivan and Shepherd, 1982; Shepherd and Sullivan, 1983), seemed to indicate that the action of an amino- or imino-containing compound was necessary for induction of germ tubes in C. albicans. We have reported (Pollack and Hashimoto, 1984) that millimolar concentrations

of ethanol, initially identified as a minor contaminant of some commercial buffer preparations, are capable of inducing germ tube formation in C. albicans. This implies that the inducer does not have to be an amino- or imino-containing compound and that competent C. albicans cells do not require any exogenous nitrogen sources for initiating germ tube formation. In previous reports of maltose or starch inducing germ tube formation, the effect was observed only in the presence of $(NH_4)_2SO_4$ (Shepherd and Sullivan, 1976) or neopeptone (Chattaway et al., 1968). Because of the potential usefulness of this system in investigating nitrogen turnover associated with the early phase of dimorphic conversion, we have further examined the conditions that critically affect ethanol-induced germ tube formation in C. albicans.

MATERIALS AND METHODS

Organism. C. albicans, ATCC 58716 was routinely used for the experiments described in this paper. In some experiments C. albicans ATCC 10261 was used for comparative purposes. Cells were maintained on Sabouraud dextrose agar (SDA, Difco Laboratories) at 25°C.

Growth and preparation of organism. Yeast phase cells grown SDA for 24 h at 37°C were collected and washed with glass distilled, deionized water ten times by vacuum filtration on a Millipore filter (0.45 μm pore size) using a Millipore filtration apparatus. To ensure complete washing, the cells were resuspended in water, before each washing, by repeated suction and forcible ejection through a pasteur pipette. The washed cells were then resuspended in distilled water and used in inoculating the induction solution.

Induction of germ tube formation. Washed C. albicans yeast-phase cells (final concentration of 2.5 to 7.5 x 10^5 cells ml^{-1}, determined turbidimetrically) were inoculated into a buffered solution (final volume, 0.5 ml) containing an inducer and the following salts: $FeSO_4$ (0.1 mg L^{-1}), KCl (400 mg L^{-1}), $MgSO_4.H_2O$ (200 mg L^{-1}), $NaH_2PO_4.H_2O$ (125 mg L^{-1}), NaCl (6400 mg L^{-1}), and $NaHCO_3$ (750 mg L^{-1}). The buffers used were sodium citrate-sodium phosphate (pH 2.5 to 6.5), HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH 7.0 to 8.0), MES (2-[N-morpholino]ethanesulfonic acid) (pH 5.5 to 6.5), MOPS (3-[morpholino]propanesulfonic acid), PIPES (1,4-piperazinediethanesulfonic acid), sodium succinate (pH 4.0 to 5.0), Tris(tris[hydroxymethyl]amino methane)-maleate (pH 5.0 to 9.0), or sodium trans-aconitate (2.5 to

6.0). However, unless indicated otherwise, Tris-maleate buffer (pH 5.8) was used. With the exception of the citrate-phosphate buffer, all buffers were adjusted to the desired pH with NaOH. The concentration of the buffer salt was 0.05 M. Unless otherwise indicated, the concentration of ethanol added to the buffered salts solution was 17.1 mM (0.1%). Dulbecco's Modified Eagle Medium (DMEM; Gibco) was used for induction in some experiments for comparative purposes after adjusting the pH value to 5.8 by dropwise addition of 0.1 M HCl, followed by vigorous shaking to remove CO_2. The cells were incubated at 37°C in a water bath for 4 h. The pH of each solution was determined at the beginning and end of each incubation with a digital Ionalyzer (Orion Research, Model 701A) using a semi-micro, combination pH/reference, electrode (No. 476050, Corning Glass Works, Medfield, MA, USA). Preliminary experiments showed that the pH did not vary more than 0.3 units during the 4 h incubation period. The percentage of cells forming germ tubes was measured by counting 100 to 200 cells in each sample with a phase contrast microscope (Nikon). Although germ tubes were generally formed from single cells, in those instances when they were formed from budded cells they were counted as one "germ tube unit" regardless of whether both the mother and daughter or only one of them had a germ tube. Germ tube lengths were measured using an ocular micrometer. Photomicrographs were taken with a Nikon M-35S camera attached to the microscope, using panchromatic film (Plus-X; Eastman Kodak).

Oxygen requirement for induction of germ tubes. To determine whether oxygen was required for germ tube formation in C. albicans cells induced by ethanol, a GasPak anaerobic system (BBL) was used. To ensure that the cells

were not inoculated into the induction solution before highly anaerobic conditions had been attained, the cell suspension was kept in a side arm attached to the test tube containing the induction solution. The tube was then secured in a rack placed in a GasPak anaerobic jar (BBL) which was then activated. After attainment of highly anaerobic conditions in the jar, as judged by the indicator system, the cells in the side arm were allowed to mix with the induction medium and then incubated at 37°C for 4 h.

RESULTS

Induction of germ tubes by ethanol

When 24 h-old yeast cells of C. albicans were incubated in buffered salts solutions containing 17.1 mM ethanol at 37° C, 70 to 80% of the inoculated cells formed germ tubes in 4 h (Table 9). Virtually no cells formed germ tubes in the absence of ethanol. All germ tubes formed in response to ethanol induction appeared somewhat thinner than those produced in rich media such as serum or DMEM (Fig. 4). The presence of bicarbonate in the incubation medium was essential for ethanol to induce these cells to form germ tubes (Table 9). Other common salts of magnesium, manganese, calcium, iron, and sodium were not required for germ tube formation.

Although most experiments reported here were done using Tris-maleate buffer, similar results were obtained when Tris-maleate was replaced by other common buffers such as sodium phosphate, citrate-phosphate, succinate, transaconitate, HEPES, MES, MOPS and PIPES. Under all conditions tested, ethanol did not induce germ tube formation in 100% of cells, whereas essentially all of the cells formed germ tubes when incubated in rich media, such as DMEM (Table 9). Ethanol-induced germ tube formation in C. albicans was not limited to strain ATCC 58716; approximately 80% of C. albicans ATCC 10261 also formed germ tubes when induced by 0.1% ethanol under similar conditions.

Optimal conditions for ethanol-induced germ tube formation

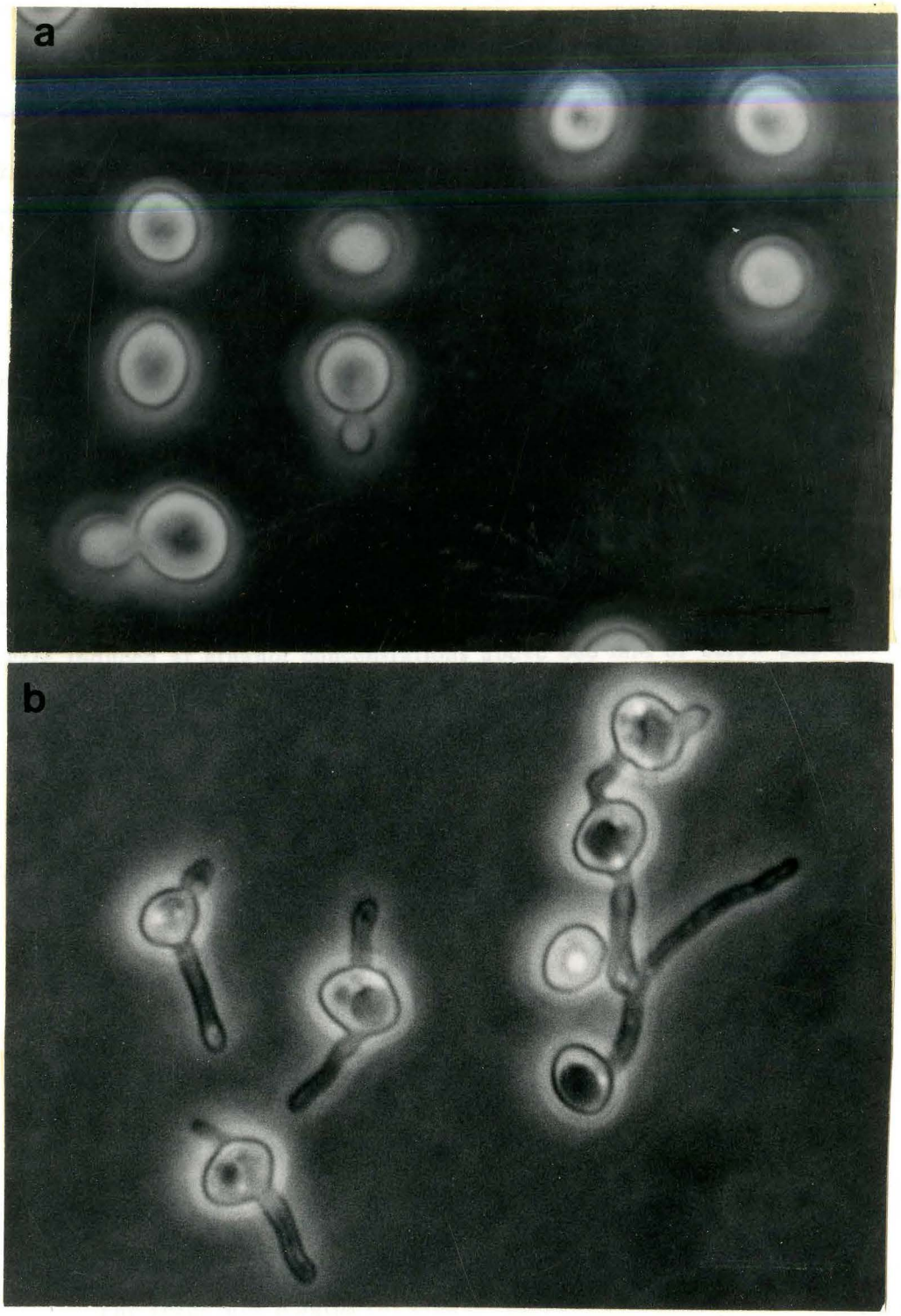
The induction of germ tubes in C. albicans by ethanol was critically affected by a number of parameters. Optimal germ tube formation (approx-

Table 9. Salt requirements for induction of germ tubes by ethanol[1]

Induction solution	Percentage of cells forming germ tubes
Buffered salts + 0.1% ethanol	77.6 ± 4.1
Buffered salts - HCO_3^- + 0.1% ethanol	23.0 ± 2.4
Buffer + HCO_3^- + 0.1% ethanol	74.2 ± 6.1
Buffer + 0.1% ethanol	17.4 ± 6.8
Buffered salts	1.9 ± 0.8
DMEM (control)	100

1 Values are the means of five independent experiments ± SEM. Composition of the buffered salts is detailed in Materials and methods. (This table is from J. H. Pollack and T. Hashimoto, 1985, J. Gen. Microbiol. 131: 3303-3310.)

Fig. 4. Phase-contrast micrographs of (a) C. albicans yeast cells grown for 24 h on Sabouraud dextrose agar, and (b) germ-tube-forming cells after 4 h induction by ethanol (0.1%) at 37°C. Bars, 10 μm. (These micrographs are from J. H. Pollack and T. Hashimoto, 1985, J. Gen. Microbiol. 131: 3303-3310.)


a
b

imately 80%) occurred at the lowest concentration of cells tested (4 x 10^5 cells ml^{-1}) The percentage of cells forming germ tubes decreased proportionately as the cell concentration increased, so that almost no cells formed germ tubes when the cell concentration was greater than 4.5 x 10^6 cells ml^{-1} (Fig. 5). The optimal temperature for germ tube formation was 37°C; however, approximately 20% of the cells formed germ tubes at either 29°C or 42°C. No cells formed germ tubes below 28°C or above 43°C (Fig. 6). Maximal germ tube formation occurred at a pH range of 5.0 to 5.8, with 40% of the cells forming germ tubes at either pH 3.0 or 7.5. Essentially no germ tubes formed below pH 2.5 or above pH 8.5 (Fig. 7). A wide range of concentrations of ethanol, from 0.34 mM to 170 mM, induced optimal or near optimal germ tube formation. Even ethanol concentrations of 0.09 mM or 340 mM induced approximately 20% of the cells to form germ tubes. However, no germ tubes were induced at ethanol concentrations above 680 mM or below 0.03 mM (Fig. 8). Since C. albicans can grow anaerobically, we tested the ability of ethanol to induce hyphal growth under strict anaerobic conditions. No germ tubes were formed under strict anaerobiosis, with ethanol or any of the other inducers tested.

Kinetics of ethanol-induced germ tube formation

Discernible germ tubes first appeared approximately 45 to 60 min after exposure to ethanol. The initial rate of germ tube formation induced by ethanol was slightly slower than that induced by DMEM. The maximum percentage of cells forming germ tubes was attained by 2 h with either ethanol or DMEM (Fig. 9).

The germ tube lengths for ethanol-induced cells drastically decreased

Fig. 5. Influence of cell density on ethanol-induced germ tube formation in C. albicans. Cells were incubated at the cell density indicated, for 4 h at 37°C, in 0.1% ethanol in buffered salts. Values are the means of three or more experiments.

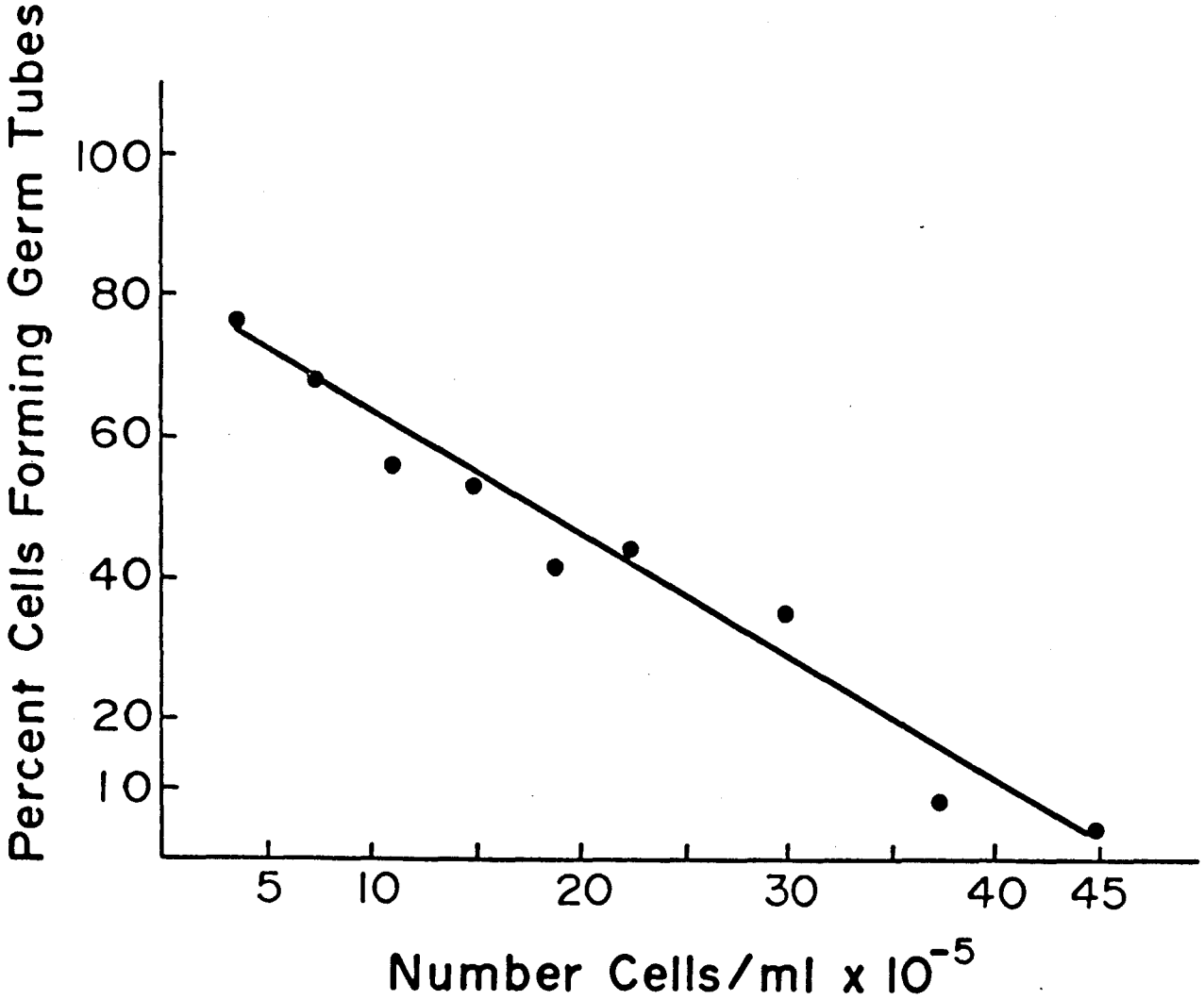

Percent Cells Forming Germ Tubes
100
80
60
40
20
10
5
10
20
30
40
45
Number Cells/ml x 10^{-5}

Fig. 6. Influence of temperature on germ tube formation induced by ethanol. Cells were incubated in 0.1% ethanol, for 4 h at the temperatures indicated. Values are the means of three experiments.

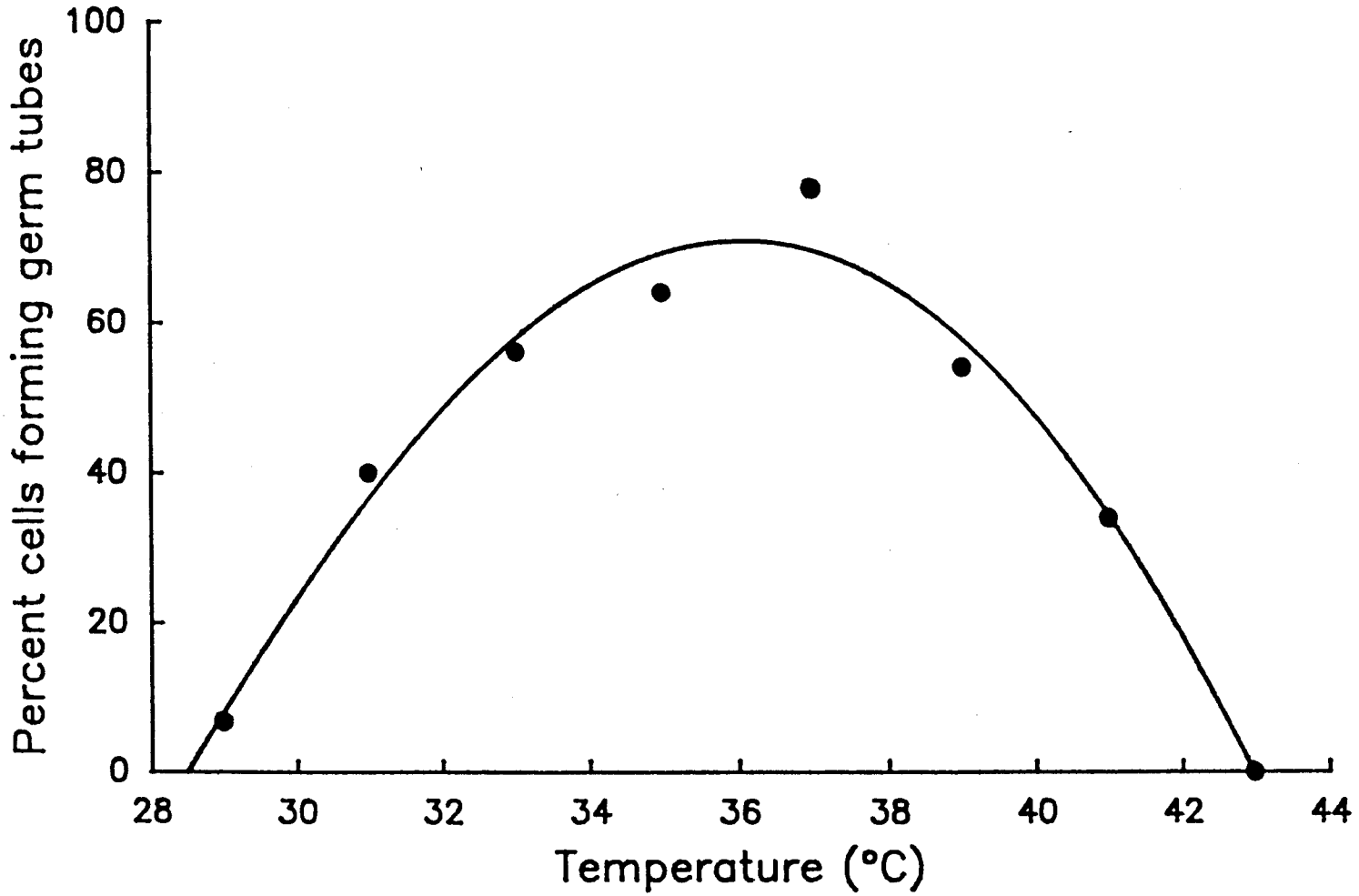

Percent cells forming germ tubes
100
80
60
40
20
0
28
30
32
34
36
38
40
42
44
Temperature (°C)

Fig. 7. Influence of pH on ethanol-induced germ tube formation in C. albicans. Cells from ATCC strain 58716 (-O-) and strain 10261 (-△-) were incubated for 4 h at 37°C in 0.1% ethanol in buffered salts at the pH values indicated. Control values for cells (strain 58716) incubated in buffered salts only are indicated by curve (-●-). Values are the means of 3 or more experiments.

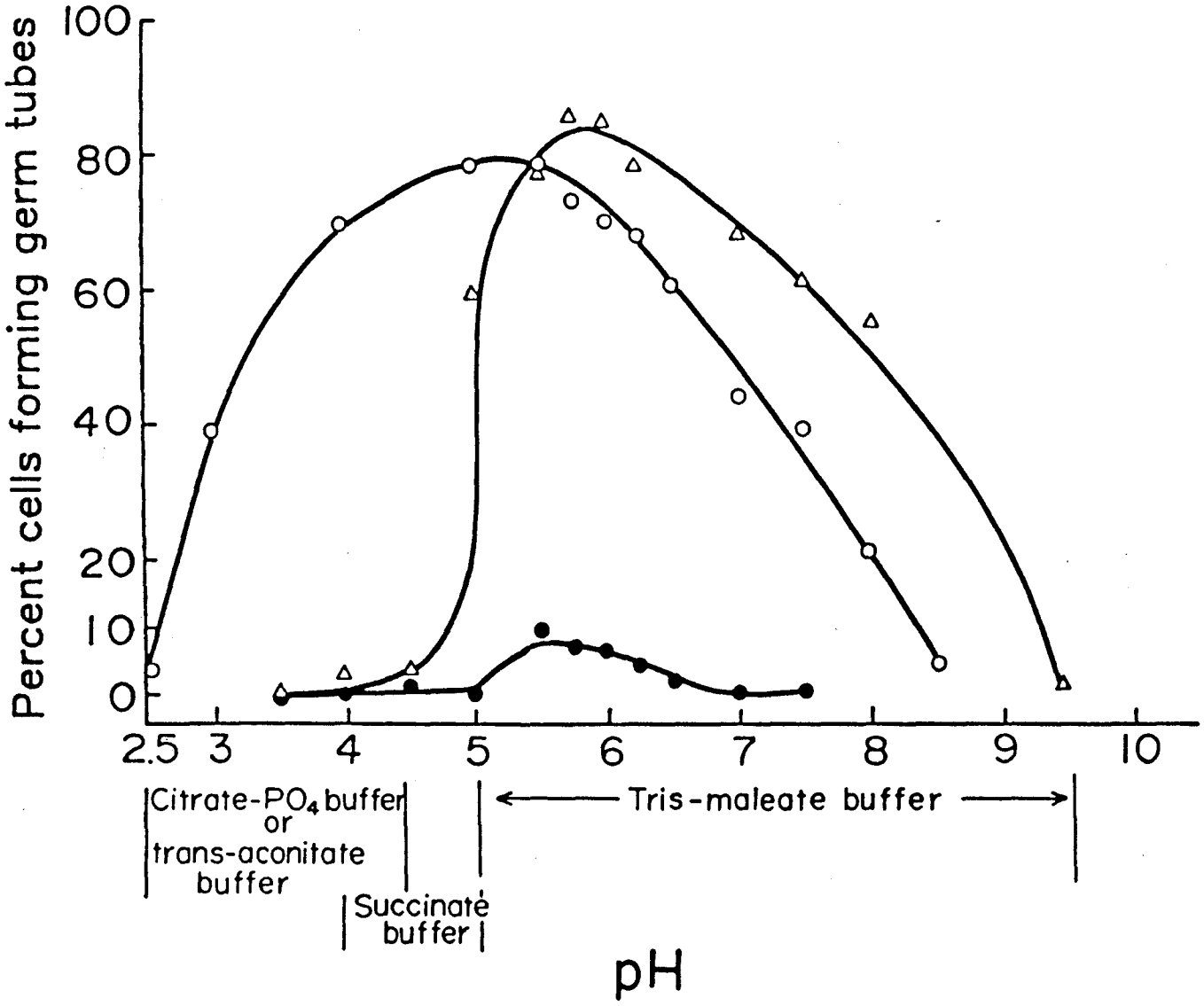

Percent cells forming germ tubes
100
80
60
40
20
10
0
2.5
3
4
5
6
7
8
9
10
Citrate-PO4 buffer
or
trans-aconitate
buffer
Succinate
buffer
Tris-maleate buffer
pH

Fig. 8. Influence of concentration of ethanol on germ tube formation induced by ethanol. Cells were incubated for 4 h at 37°C in buffered salts containing the indicated amount of ethanol. Values are the means of 4 or more experiments.

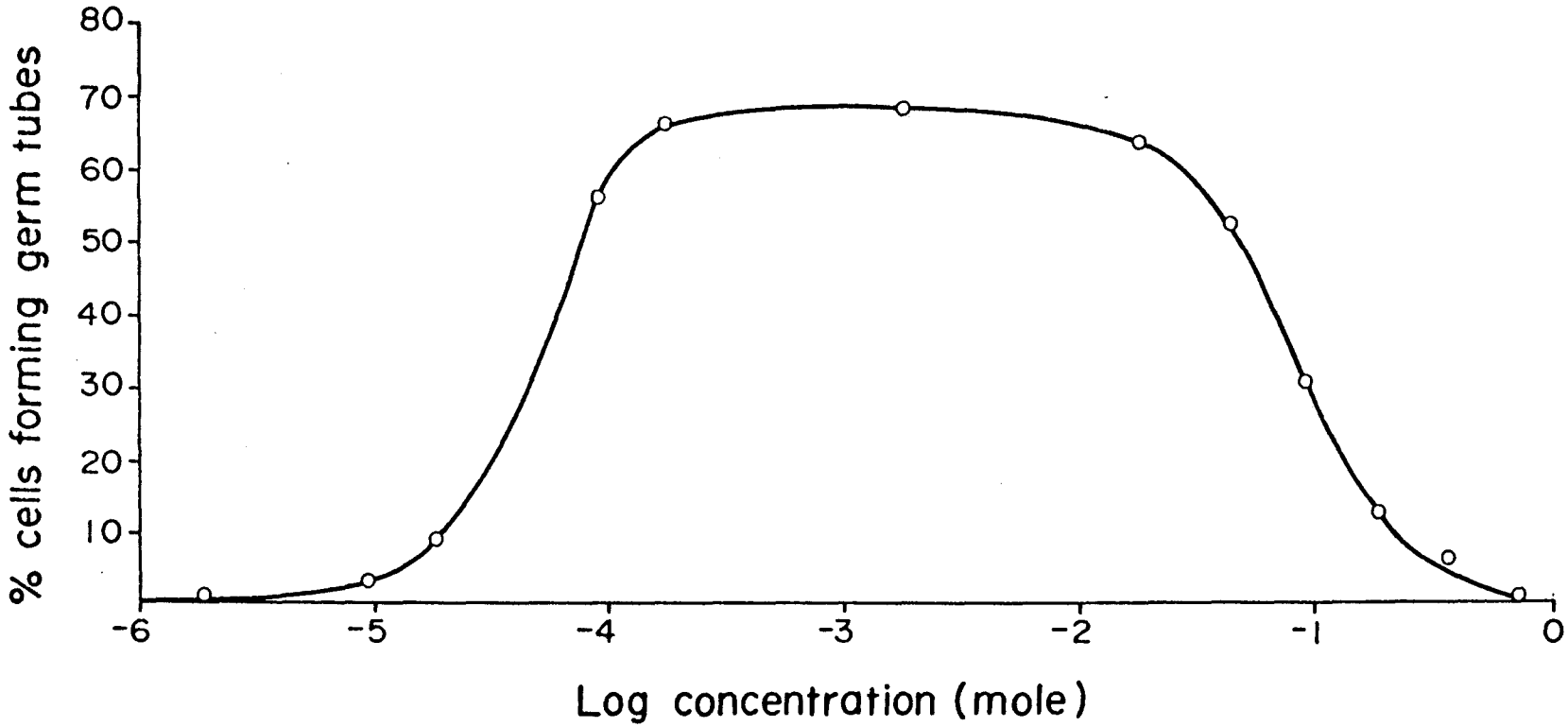
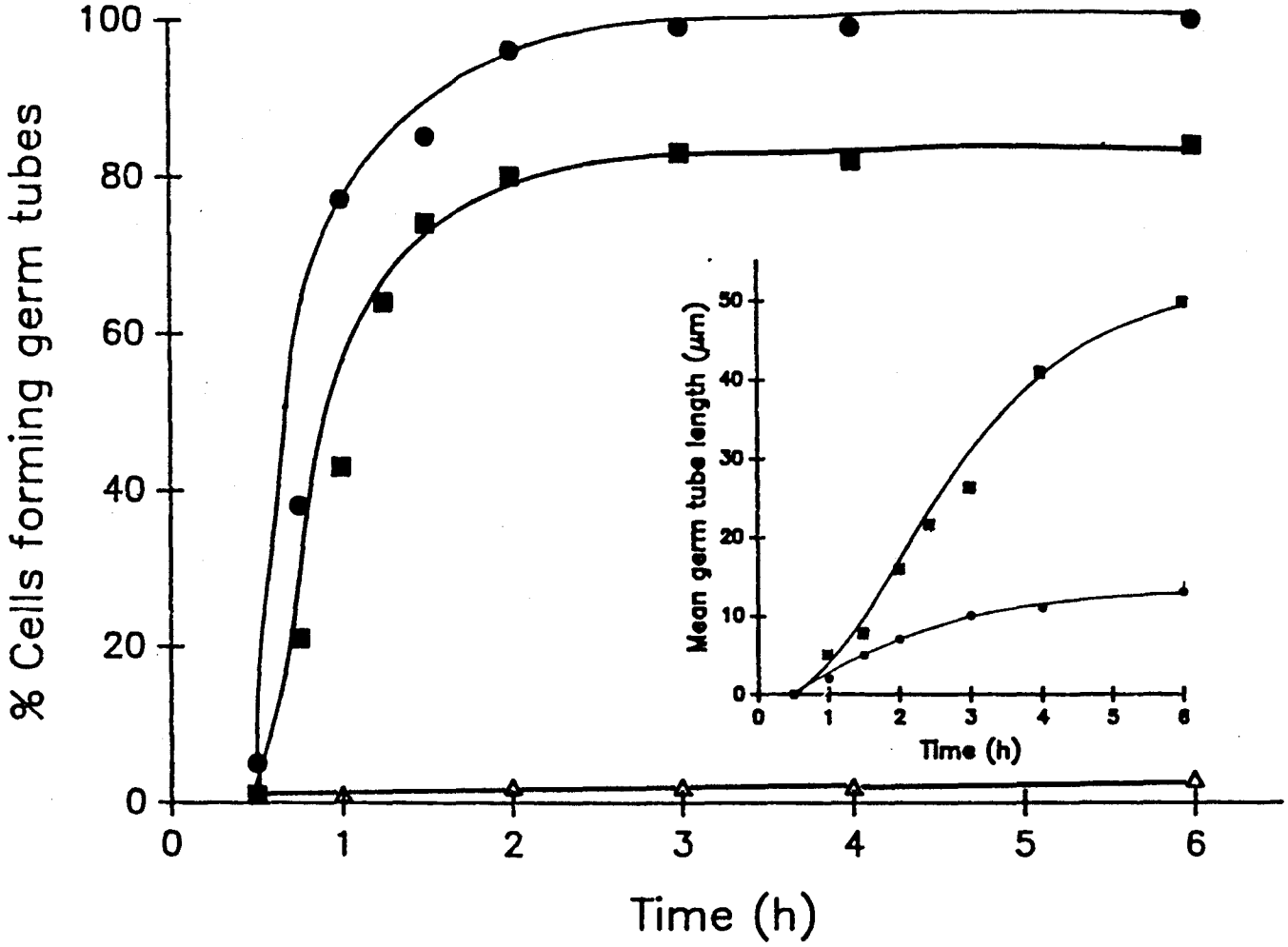

% cells forming germ tubes
80
70
60
50
40
30
20
10
-6
-5
-4
-3
-2
-1
0
Log concentration (mole)

Fig. 9. Kinetics of germ tube formation. Cells were induced to form germ tubes at pH 5.8 and 37°C either by DMEM (●) or by 0.1% ethanol in buffered salts solution (■). No germ tubes were formed in buffered salts solution in the absence of ethanol (△). The mean length of the germ tubes formed at each interval is indicated in the inset. The average length of 50 germ tubes formed at each interval is indicated in the insert. Values are the result of a representative experiment. (This figure is from J. H. Pollack and T. Hashimoto, 1985, J. Gen. Microbiol. 131: 3303-3310.)


% Cells forming germ tubes
100
80
60
40
20
0
0
1
2
3
4
5
6
Time (h)
Mean germ tube length (μm)
50
40
30
20
10
0
0
1
2
3
4
5
6
Time (h)

as the pH of the medium was increased above 6.0. At pH 5.8 the longest germ tubes were approximately 30 μm long, the mean length being about 11 μm after 6 h. There were no significant differences in the mean germ tube length when the concentration of ethanol was increased from 0.001% (0.17 mM) to 1.0% (171 mM) (Table 10).

Other compounds capable of hyphal induction

Of all the alcohols tested, only 1-propanol, 2-propanol, and 1-butanol were capable of inducing germ tubes. None of these alcohols was able to induce as high a percentage of cells to form germ tubes as ethanol (Table 11). Methanol, 2-butanol, 3-butanol, 1-pentanol, 2-pentanol, phenylethyl alcohol, acetone, methylethyl ketone, dimethylsulfoxide, polyethylene glycol 400 and 6000, Triton-X 100 and acetaldehyde could not induce germ tube formation in strain ATCC 58716 of C. albicans. However, 0.1% acetic acid was also capable of inducing 50% of the cells to form germ tubes.

Alcohol dehydrogenase involvement in ethanol-induced germ tube formation

To determine whether metabolism of ethanol by alcohol dehydrogenase (ADH) is necessary for germ tube formation, we tested the effect of adding allyl alcohol and pyrazole, an inhibitor of yeast ADH (Reynier, 1969). Allyl alcohol is converted to the lethal compound acrolein, in the presence of ADH. However, the conversion of allyl alcohol to acrolein is inhibited by pyrazole, allowing the cells to grow (Paz et al., 1984). Allyl alcohol (0.05 mM to 50 mM) and pyrazole (0.5 mM to 50 mM) were added to the induction solution (0.0625 ml, final volume) containing either ethanol (0.1%) or GlcNAc (4 mM) as the inducer. The percentage of cells forming germ tubes was assessed af-

Table 10. Effect of concentration of ethanol on germ tube length.[1]

Ethanol conc. (%, v/v)	Average germ tube length (μm ± SD) at pH 5.8	7.2
0	0	0
0.001	13.6 ± 5.7	4.2 ± 2.4
0.01	15.2 ± 9.3	4.2 ± 2.6
0.1	16.9 ± 8.4	5.1 ± 2.7
DMEM (control)	N.D.	52.4 ± 23.2

1. Values are the average length ± SD of 50 germ tubes formed at each condition.

Table 11. Induction of germ tubes by higher alcohols and related compounds.

Compound	Percentage of cells forming germ tubes at an inducer concentration (%, v/v) of:[1]			
	0.5	0.1	0.01	0.001
1-Propanol	20.2 ± 7.1	51.2 ± 3.6	48.2 ± 5.0	22.8 ± 4.5
2-Propanol	36.5 ± 9.1	49.2 ±4.0	43.5 ± 7.1	8.0 ± 4.4
1-Butanol	3.5 ± 0.9	23.0 ± 5.4	24.8 ± 6.5	10.2 ± 2.8
Acetic acid	0	49.0 ± 3.0[2]	20.5 ± 18.5[2]	0

1 Values are means ± SEM for at least four independent experiments. (This table is from J. H. Pollack and T. Hashimoto, 1985, J. Gen. Microbiol. 131: 3303-3310.)

2 Mean ± the range of two experiments.

ter 6 h.

The ability of pyrazole to inhibit the lethal action of allyl alcohol could be demonstrated only if C. albicans was induced to form germ tubes by GlcNAc. This ability was dependent on the concentration of both pyrazole and allyl alcohol (Table 12). As the concentration of allyl alcohol in the induction solution was increased, a similar increase in the concentration of pyrazole was required to overcome the lethal effects. In all instances where a lethal concentration of allyl alcohol (0.5 mM) was added, ethanol could not induce germ tube formation regardless of the concentration of pyrazole. Indeed, pyrazole itself was inhibitory to ethanol-induced, but not to GlcNAc-induced, germ tube formation, at a concentration greater than 5 mM. However, pyrazole alone, at 1 mM, a concentration sufficient to overcome the lethal effects of 0.5 mM allyl alcohol in the GlcNAc-inducing system, did not significantly affect the germ tube-inducing capacity of ethanol. Thus, while germ tube induction by ethanol may be possible even when ADH is inhibited, these results are not conclusive.

Table 12. Germ tube formation in the presence of pyrazole and allyl alcohol.[1]

Inhibitor	Percentage of cells forming germ tubes when induced by:	
	Ethanol	GlcNAc
None	77	100
Allyl alcohol (0.5 mM)	0	0
Pyrazole (10 mM)	11	100
Pyrazole (5 mM)	38	100
Pyrazole (1 mM)	65	100
Pyrazole (0.5 mM)	71	100
Allyl alcohol (0.5 mM) + pyrazole (0.5 mM)	0	35
Allyl alcohol (0.5 mM) + pyrazole (1 mM)	3	100
Allyl alcohol (5 mM) + pyrazole (5 mM)	0	13
Allyl alcohol (5 mM) + pyrazole (10 mM)	0	88
Allyl alcohol (50 mM) + pyrazole (20 mM)	0	0
Allyl alcohol (50 mM) + pyrazole (50 mM)	0	62

1 Allyl alcohol and/or pyrazole were added to the buffered salts solution described in Material and Methods, containing either ethanol (17.1 mM) or GlcNAc (4 mM) as the inducer. The final volume in each tube was 0.625 ml. (This table is from J. H. Pollack and T. Hashimoto, J. Gen. Microbiol. 131: 3303-3310.)

DISCUSSION

One of the most perplexing aspects of germ tube induction by ethanol is that, under the optimal conditions found, ethanol can induce at most 85% of the cells to form germ tubes. On the other hand GlcNAc, L-proline of DMEM consistently induce essentially all of the cells to form germ tubes. This effect may be due to each cell in the population being required to arrive at some critical point in the cell cycle in order for hyphal formation to ensue. Although a number of investigators have shown that under given conditions, cells from all growth phases are capable of being induced (Mattia and Cassone, 1979; Ahrens et al., 1983; Soll and Herman, 1983), it is still possible that their conditions actually bring the cells to a critical cell cycle point when the cells are "pluripotent". Thus, while other inducers are rich enough nutrients to bring every cell in an asynchronous population to this critical point, ethanol may be too poor a nutrient source to accomplish this. Hence, a certain percentage of cells would remain ungerminated.

The possible lack of sufficient storage reserves in each cell of the population, or of the ability to utilize them,, may also account for the fact that less than 100% of the cells formed germ tubes. Both trehalase and glucoamylase are induced by ethanol in Saccharomyces cerevisiae (Panek, 1962; Rambeck and Simon, 1972) and mobilization of glycogen does seem to correlate with germ tube formation by C. albicans (Ram et al., 1984; Sullivan et al, 1984). Recent reports on proteases emphasized their possible roles in the pathogenicity of C. albicans, but have not considered the possibility that the breakdown of cytoplasmic materials or wall glycoproteins may also be integrally involved in germ tube formation. We have yet to show a relationship

between ethanol and mobilization of carbohydrate or nitrogen reserves in C. albicans, yet it would seem very likely that such mobilization is necessary for germ tube formation to occur. Carbohydrate reserves probably are necessary to supplement the small concentration of ethanol required to induce germ tube formation, and it is certain that all nitrogen must be obtained endotrophically as there is no exogenous nitrogen in the induction solution. Thus, differences in a certain percentage of cells in a population able to respond to ethanol may be attributed to differences in their endogenous reserves.

The basis of the requirement of bicarbonate for the effective induction of germ tubes by ethanol (Table 9) is not understood, especially since this requirement was eliminated if the cells were starved by incubating them in water at 20°C for 24 h before induction (unpublished observations). There is a possibility that bicarbonate may be required for the metabolism of ethanol, or that it may increase the permeability of the cell membrane to ethanol, as has been suggested in the case of dimorphism in Mucor (Stewart and Rodgers, 1983). It does seem unlikely, though, that bicarbonate is an initiator of dimorphism in C. albicans (Mardon et al., 1969) since it is not universally required.

While the pH values at which ethanol induced germ tube formation were considerably below those reported for other systems (Simonetti et al., 1974; Evans et al., 1975; Mitchell and Soll, 1979), replacing ethanol with GlcNAc, proline or DMEM in our system gave similar results. Thus it would seem that the composition of the induction medium plays a major role in the sensitivity to pH.

The recent evidence that a metabolizable inducer is not required for germ tube induction (Sullivan and Shepherd, 1982; Shepherd and Sullivan,

1983), prompted us to check if ethanol metabolism is required for germ tube induction. Our attempts to clarify this issue using pyrazole, an inhibitor of yeast ADH (Reynier, 1969), were inconclusive. A concentration of pyrazole (1 mM) capable of inhibiting the lethal action of a low concentration of allyl alcohol, and presumably of inhibiting ADH, was found, suggesting the possibility that ethanol metabolism via ADH is not required. However, this inhibitory action of pyrazole could not be demonstrated in the presence of allyl alcohol in an ethanol-induced system, for unknown reasons. It is possible that sufficient ADH remained active in the presence of 1 mM-pyrazole to metabolize whatever ethanol was necessary for germ tube formation. It is not possible to argue that higher concentrations of pyrazole were inhibitory because they more severely inhibited ADH, since imidazole, an isomer of pyrazole which does not counteract the effects of allyl alcohol, also inhibits germ tube induction (data not shown), presumably by a mechanism other than inhibition of ADH. However, even if ADH is not required for ethanol-induced germ tube formation, this only eliminates one route for ethanol metabolism. Alcohol can also be metabolized oxidatively by a microsomal ethanol oxidizing system in rats (Lieber and De Carli, 1968) and non-oxidatively by a esterification with fatty acids (Lange, 1982). Thus we cannot yet exclude the possibility that ethanol must be metabolized for germ tube induction to occur. The fact that increasing concentrations of ethanol did not result in significantly longer germ tubes might be explained by the fact that the endogenous nitrogen reserves limited the ultimate germ tube length.

Although propanol and butanol could also induce germ tube formation, the results would seem to indicate that the effect of ethanol is not due to a non-specific interaction with the cell membrane, as has been suggested for

ethanol-induced germ tube dimorphism in Aureobasidium pullulans (Sevilla et al., 1983), since methanol and other membrane-disturbing compounds were completely ineffective in inducing germ tube formation in C. albicans. As it is possible that methanol cannot be metabolized by C. albicans, and thus germ tube induction would not be recognized, we did attempt to induce germ tubes with methanol in combination with glucose and other sugars and amino acids. No germ tubes were formed unless methanol was combined with an amino acid which could induce germ tubes by itself (data not shown). Thus, it appears that the ethanol effect in C. albicans is more specific than thought to occur in A. pullulans.

CHAPTER IV

THE ROLE OF GLUCOSE IN THE pH REGULATION OF GERM TUBE FORMATION IN CANDIDA ALBICANS

ABSTRACT

It has been reported that Candida albicans can form germ tubes only in the narrow pH range of 6-8, and that by changing only the pH, one can regulate germ tube formation. The pH minimum for germ tube formation could be dramatically lowered by eliminating the glucose present in many induction solutions. Lee's medium, lacking glucose, ethanol, N-acetylglucosamine, and proline, induced germ tubes at pH values as low as 3 under most conditions. The presence of as little as 1 mM glucose in these induction solutions was sufficient to cause the cells to grow either as yeasts with multiple-buds or as pseudohyphae when the pH was 3.7. However, when C. albicans was grown in any of the above induction solutions, with the exception of ethanol, containing 200 mM glucose buffered at pH 5.8, not only were germ tubes formed, but the rate of germ tube formation and germ tube length were increased. Preincubation of the cells in a solution buffered at pH 3.7 containing 200 mM glucose, prior to exposure to induction solutions lacking glucose at pH 3.7 or at pH 5.8, did not inhibit germ tube formation. Likewise, addition of glucose after 45 min exposure to an induction solution was without effect. Theophylline and dibutryl cAMP did not counteract the

This chapter is, in part, from J. H. Pollack and T. Hashimoto, 1987, J. Gen. Microbiol., 133: 414-424.

action of glucose. Other sugars which suppressed germ tube formation at low pH were fructose, galactose, mannose, xylose, gluconic acid and the non-metabolizable sugar, 3-O-methylglucose. These results indicate that pH does not directly regulate dimorphism in C. albicans, and that glucose or its metabolites may play an important role in the control of C. albicans dimorphism.

INTRODUCTION

A number of factors have been known to regulate the dimorphic transformation of Candida albicans from budding yeast cells to elongating hyphae. Among these, the most important factors have been the growth medium and temperature. Specifically, when C. albicans yeast cells are transferred to a medium containing either certain amino acids, such as proline, or N-acetylglucosamine (GlcNAc) or ethanol, and incubated at a temperature between 33 and 42°C, they are induced to grow by forming germ tubes which elongate to form hyphae.

Although many groups have reported that germ tube formation is restricted to the narrow pH range of 6 to 8 (Odds, 1979), only recently has pH been advanced as an equally important "regulator" of C. albicans dimorphism. Buffo et al. (1984) have presented evidence that as long as the pH value of a medium allowing germ tube formation is kept above 6, C. albicans grows in the hyphal form. Should the pH drop below 6 the cells revert to growing as a budding yeast.

Using ethanol as an inducer of germ tube formation (Pollack & Hashimoto, 1985), we have shown that germ tubes could form even when the pH was as low as 3.0. This led us to look for an explanation for the differences between our findings and those of Soll and co-workers (Mitchell & Soll, 1979, Buffo et al., 1984).

This section attempts to show that it is glucose, present in many media, including that of Lee et al. (1975) used by Soll and co-workers (Mitchell and Soll, 1979; Buffo et al., 1984), which suppresses germ tube formation when the pH is below 5.8, and that, therefore, pH can at most be considered

an "indirect" regulator of germ tube formation.

METHODS

Organism. C. albicans, ATCC 58716 was routinely used for the experiments described in this paper. In some experiments C. albicans ATCC 10261 was used for comparative purposes. Cells were maintained on Sabouraud dextrose agar (SDA, Difco Laboratories) at 20°C.

Growth and preparation of organism. Yeast phase cells were prepared by growing the cells on SDA for 24 h at 37°C. In some experiments cells were also prepared by growing them for 24 h at 37°C in Sabouraud dextrose broth (SDB) containing 0.1% yeast extract, or by growth in the medium of Lee et al. (1975)(referred to as "Lee's medium" or LM) for 48 to 72 h at 23°C as described by Buffo et al. (1984). The cells were collected and washed with glass distilled, deionized water ten times by vacuum filtration on a Millipore filtration apparatus, with the cells being resuspended in water by repeated suction and forcible ejection through a pasteur pipette before each washing.

Induction of germ tube formation. Washed C. albicans yeast-phase cells (final concentration of 3×10^6 cells ml^{-1}) were inoculated into a buffered solution (final volume, 0.5 ml) containing an inducer and the following salts: $FeSO_4$ (0.1 mg L^{-1}), KCl (400 mg L^{-1}), $MgSO_4.H_2O$ (200 mg L^{-1}), $NaH_2PO_4.H_2O$ (125 mg L^{-1}), NaCl (6400 mg L^{-1}), and $NaHCO_3$ (750 mg L^{-1}). The buffers used were sodium citrate-sodium phosphate (pH 2.5 to 6.5), HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH 7.0 to 8.0), MES (2-[N-morpholino]ethanesulfonic acid) (pH 5.5 to 6.5), sodium succinate (pH 4.0 to 5.0), Tris(tris[hydroxymethyl]amino methane)-maleate (pH 5.0 to 9.0), trans-

aconitate (2.5 to 6.0), or TAPS(tris[hydroxymethyl]methylaminopropanesulfonic acid)(pH 7.5 to 9.5). With the exception of citrate-phosphate buffer all buffers were adjusted to the desired pH with NaOH. The concentration of the buffer salt was 0.05 M. The inducers used were N-acetyl-D-glucosamine (GlcNAc), L-proline, at 4 mM, the combination of glucose and NH_4Cl, each at 5 mM, ethanol at 17.1 mM (0.1% v/v), or a solution containing the amino acids (L-alanine,5.6 mM; L-leucine, 9.9 mM; L-lysine, 5.5 mM; L-methionine, 0.7 mM; L-phenylalanine, 3.0 mM; L-proline, 4.3 mM; and L-threonine,4.2 mM) found in LM (referred to as "Lee's amino acids" or LAA). The cells were incubated at 37°C in a water bath for 4 h. The pH of each solution was determined at the beginning and end of each incubation with a digital Ionalyzer (Orion Research, Model 701A) using a semi-micro, combination pH/reference, electrode (No. 476050, Corning Glass Works, Medfield, MA, USA). Preliminary experiments showed that the pH did not vary more than 0.3 units during the 4 h incubation period. The percentage of cells forming germ tubes or multiple buds was measured by counting 100 to 200 cells (or cell clusters) in each sample with a phase contrast microscope (Nikon). A cell was considered multi-budded if two or more, round or ellipsoidal (pseudohyphal) buds were attached to the mother cell, while a cell was considered to have formed a germ tube when a narrow tube, at least 2 m long, without a constriction at its base, extended from the mother cell. Each cluster of cells was considered as one unit so that 50 multi-budded clusters in a population containing an additional 50 single cells would mean that 50% of the population was multi-budded. Although germ tubes were generally formed from single cells, in those instances when they were formed from budded cells they were counted as one "germ tube unit" regardless of whether both the mother and daughter or only

one of them had a germ tube. Germ tube lengths were measured using an ocular micrometer.

Chemicals. Most of the sugars used in this study were obtained either from Nutritional Biochemicals Corp. or Sigma Chemical Co. The exceptions were N-acetylglucosamine, glucosamine, sodium glucoheptonate and gulono-1,4,-lactone (Pfanstiel), glucose and glycerol (Mallinckrodt), and lactose (J.T. Baker). HEPES, MES, TAPS, amino acids and dibutryl cyclic AMP were obtained from Sigma, theophylline from Calbiochem and the salts used in the buffered salts solution from J.T. Baker. All chemicals were used without further purification.

RESULTS

Influence of pH on induction of germ tubes by different inducers

The range of pH values which allowed germ tube formation was tested using a number of different inducers. The results shown in Fig. 10 demonstrate that germ tubes were induced in C. albicans by proline when the pH was between 3 and 9. Almost identical results were obtained when proline was replaced by LAA or GlcNAc. If ethanol replaced proline a similar result was obtained with the exception that the maximum percent of cells forming germ tubes was approximately 85% (See Pollack and Hashimoto, 1985). Decreasing the concentration of inducer 100-fold resulted in only a partial decrease in the percent of cells forming germ tubes at the pH extremes. The pH range at which germ tube formation occurred was not altered either by increasing the concentration of proline or GlcNAc 100-fold. In other words, there apparently is no sharply defined pH optimum for germ tube formation. The addition of 200 mM glucose to LAA (Fig. 11) or the other inducers (Table 13), suppressed germ tube formation when the pH was less than 5.8. The addition of glucose to ethanol-induced cells, however, suppressed germ tube formation even above pH 5.8. Up to 90% of the cells which normally formed germ tubes at pH 3.7 were inhibited from doing so by the presence of glucose (Table 13). These results were not unique to ATCC strain 58716, but were also confirmed using ATCC strain 10261. In addition, since the cells used by Buffo et al. (1984) were grown initially in LM, yeast cells were prepared as they reported, and also by growth in Sabouraud dextrose broth containing 0.1% yeast extract for 24 h. These yeast cells were also tested for their ability to form germ tubes at different pH values. The results (not shown)

Fig. 10. Influence of pH on germ tube formation. C. albicans yeast cells were incubated for 4 h at 37°C in buffered salts solution (-●-) or buffered salts solutions containing 4 mM proline (-O-). For buffers used to control the pH, see Methods. Values are the means of at least 4 independent experiments. Bars represent SD. (This figure is from J. H. Pollack and T. Hashimoto, 1987, J. Gen. Microbiol. 133: 415-424.)

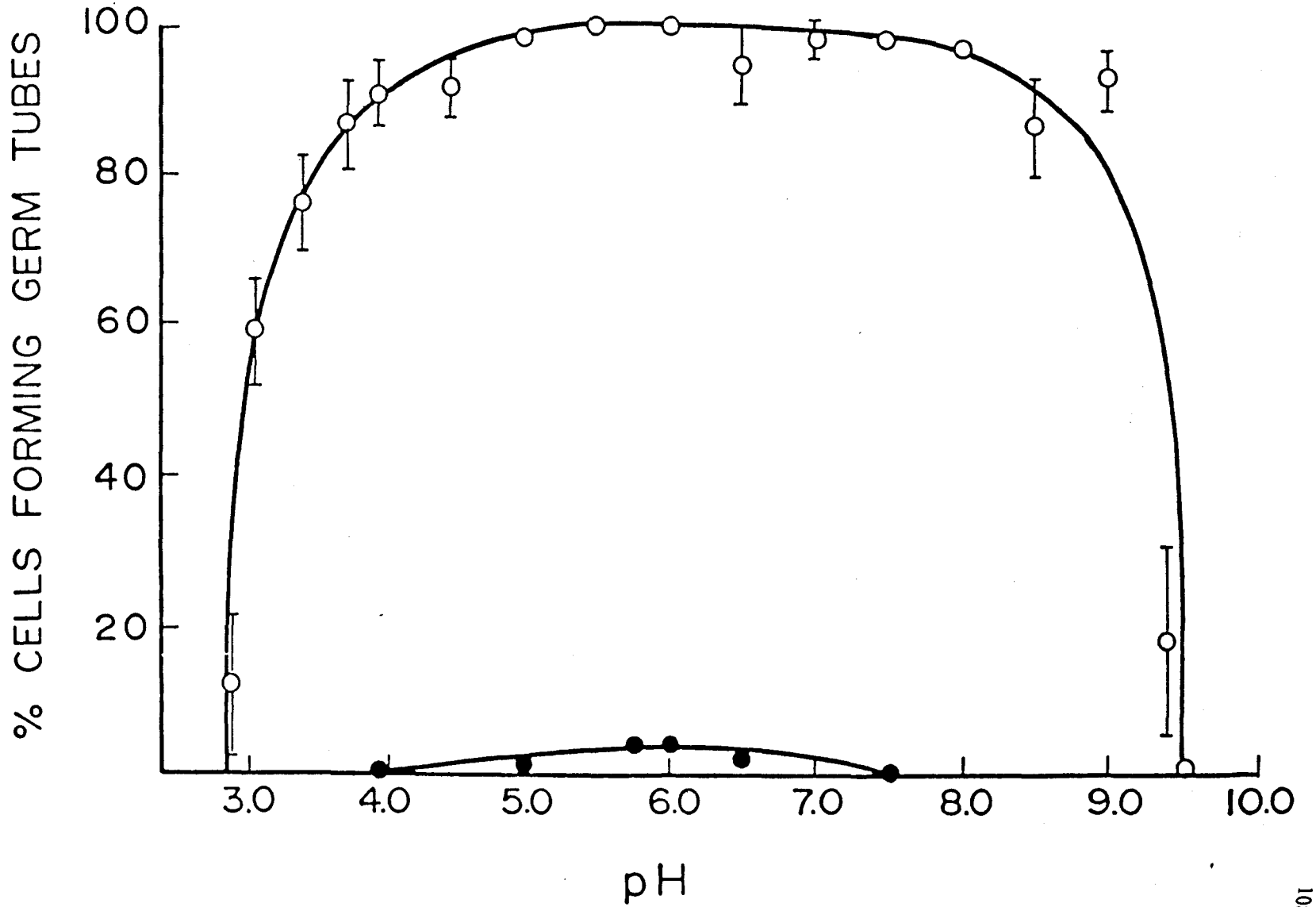

% CELLS FORMING GERM TUBES
100
80
60
40
20
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
pH

Fig. 11. Influence of glucose on germ tube and multiple bud formation at different pH values. C. albicans yeast cells were incubated for 4 h at 37° C in buffered salts solution containing LAA in the presence (-●-, -■-) or absence (-O-) of 200 mM glucose. Similar curves were obtained if LAA was replaced by proline or GlcNAc. A curve similar to (-●-) was also obtained if the cells were induced by the combination of glucose and NH_4Cl, each at 5 mM. (-O-, -●-), Cells forming germ tubes; (-■-), cells forming multiple buds. The values are the means of 4 independent experiments. Bars represent SD. (This figure is from J. H. Pollack and T. Hashimoto, 1987, J. Gen. Microbiol. 133: 415-424.)

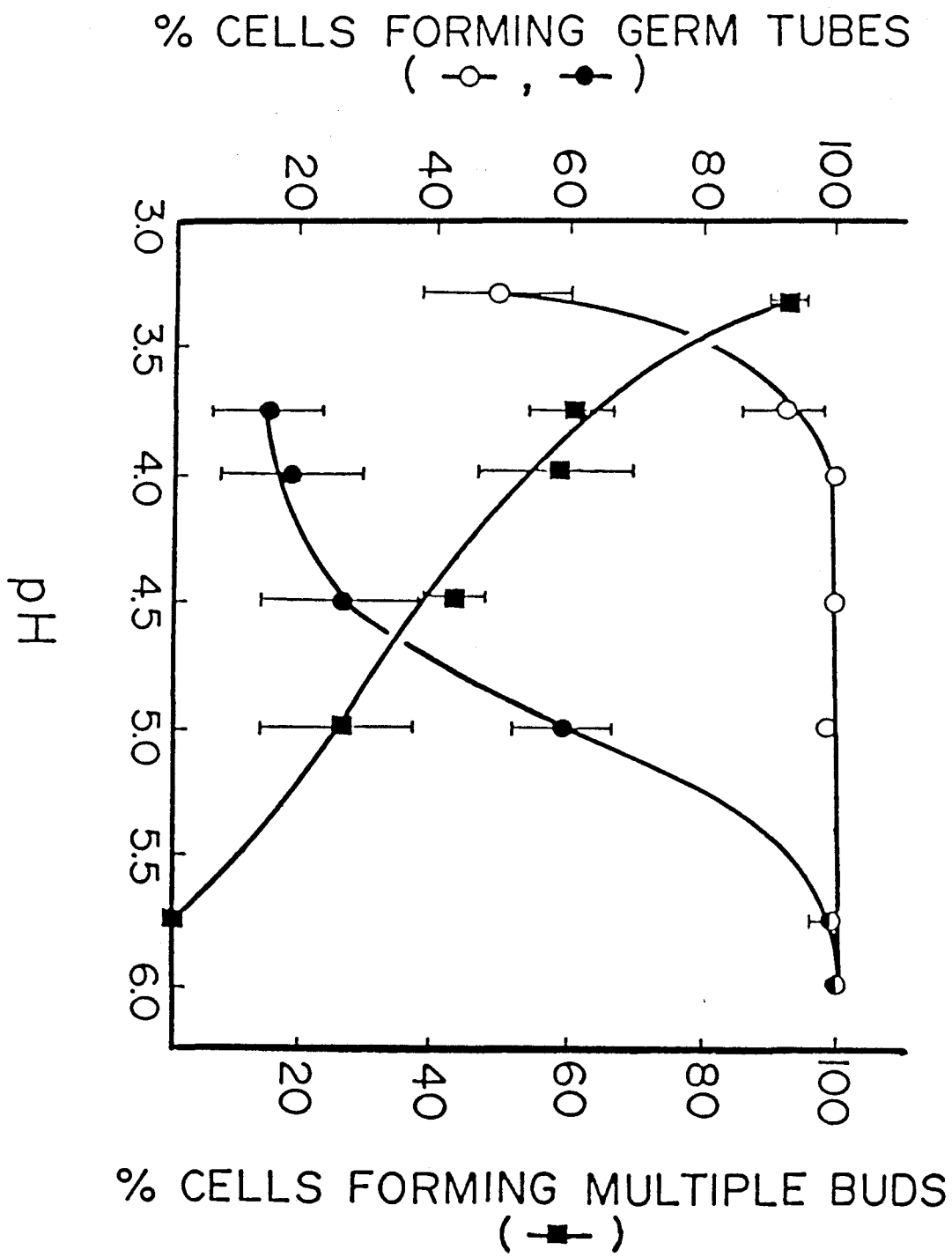

% CELLS FORMING GERM TUBES
(-o- , -●-)
20
40
60
80
100
3.0
3.5
4.0
4.5
5.0
5.5
6.0
pH
20
40
60
80
100
% CELLS FORMING MULTIPLE BUDS
(-■-)

Table 13. Suppression by glucose of germ tube formation induced by different compounds.

Inducer[1]	Percent Cells Forming Germ Tubes at Low pH, Glucose Concentration of:	
	0 mM	200 mM
LAA[2]	92.3 ± 6.9	12.0 ± 8.0
Proline	88.2 ± 5.8	10.7 ± 7.1
GlcNAc[3]	93.5 ± 4.6	21.2 ± 7.2
Ethanol	66.6 ± 9.5	8.8 ± 8.0

1 Cells were incubated as described in Methods at pH 3.7 (except for ethanol, at pH 4.3) in the presence or absence of 200 mM glucose. Values represent the means of 4 to 7 independent experiments ± the standard deviation (SD). (This table is from J. H. Pollack and T. Hashimoto, 1987, J. Gen. Microbiol. 133: 415-424.)

2 "Lee's amino acids". See Methods.

3 N-acetylglucosamine

were essentially identical to those obtained using yeast cells grown on SDA.

Concentration of glucose necessary for suppression of germ tube formation

The concentration of glucose in LM is fairly high (70 mM). We therefore tested the cells in the different induction media for the minimum concentration of glucose required to suppress germ tube formation. The results (Fig. 12) show that even 1 mM glucose was capable of suppressing germ tube formation at pH 3.7. Increasing the concentration of glucose resulted in enhanced suppression of germ tube formation; however, even at the highest concentration of glucose tested (400 mM), germ tube formation was never completely inhibited. Approximately 10% of the cells were still capable of forming germ tubes. Suppression was considerably greater at all concentrations of glucose tested, except 400 mM, when the cells were induced by LAA or proline than when they were induced by GlcNAc. While germ tube formation induced by LAA was suppressed approximately 67% and 84% by 5 mM and 200 mM, respectively, at pH 3.7, it was only suppressed 18% and 76% when induced by GlcNAc. Increasing the pH to 5.0 required greater amounts of glucose to obtain suppression than at pH 3.7 (data not shown).

Appearance of cells grown at low pH in the presence of glucose

Yeast cells grown in non-germ-tube-inducing conditions (i.e. on SDA or in LM at room temperature) generally formed single buds which separated from the mother cell before another bud was formed. Occasionally 2 buds could be seen attached to the mother cell. In contrast, cells grown in glucose at low pH tended to grow as pseudohyphae or as chains of yeast cells

Fig. 12. Effect of increasing the glucose concentration on germ tube and multiple bud formation in C. albicans yeast cells induced to form germ tubes at pH 3.7. Cells were incubated for 4 h at 37°C in buffered salts solution containing LAA (-●-, -■-) or GlcNAc (-O-). Curves essentially identical to (-●-) and (-■-) were obtained when LAA was replaced by proline. (-O-, -●-), Cells forming germ tubes; (-■-), cells forming multiple buds. Values are the means of 4 independent experiments. Bars represent SD. (This figure is from J. H. Pollack and T. Hashimoto, 1987, J. Gen. Microbiol. 133: 415-424.)

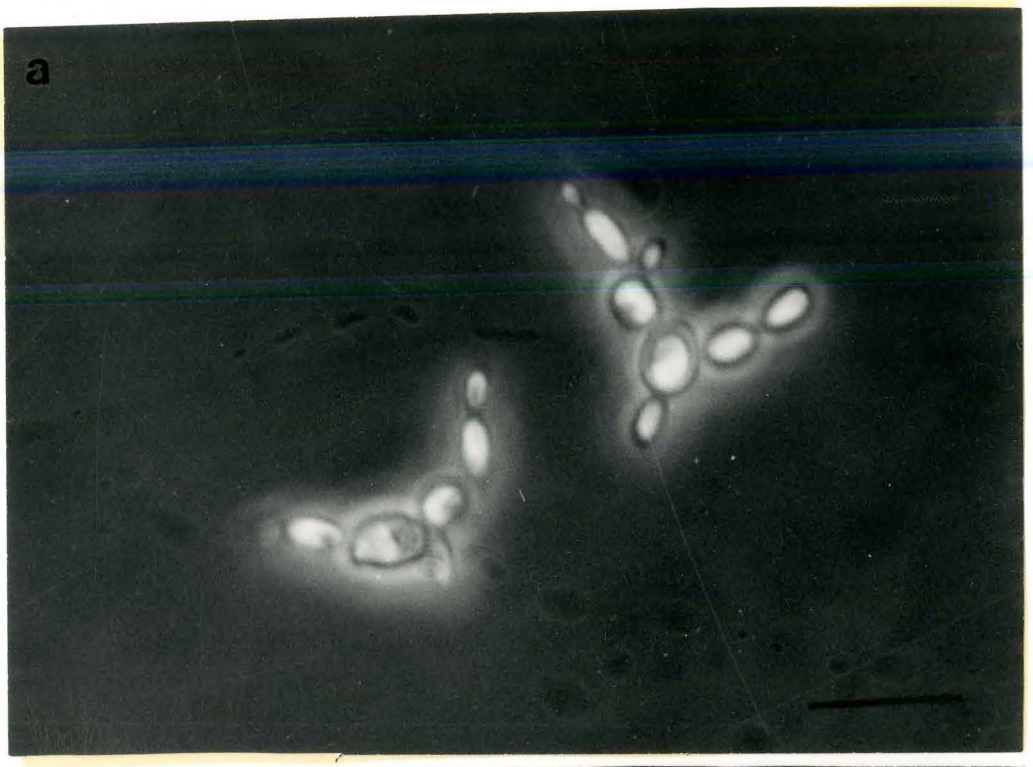
% CELLS FORMING GERM TUBES
(—○— , —●—)
% CELLS FORMING MULTIPLE BUDS
(—■—)
100
80
60
40
20
0
20
50
100
150
200
400
GLUCOSE CONCENTRATION (mM)

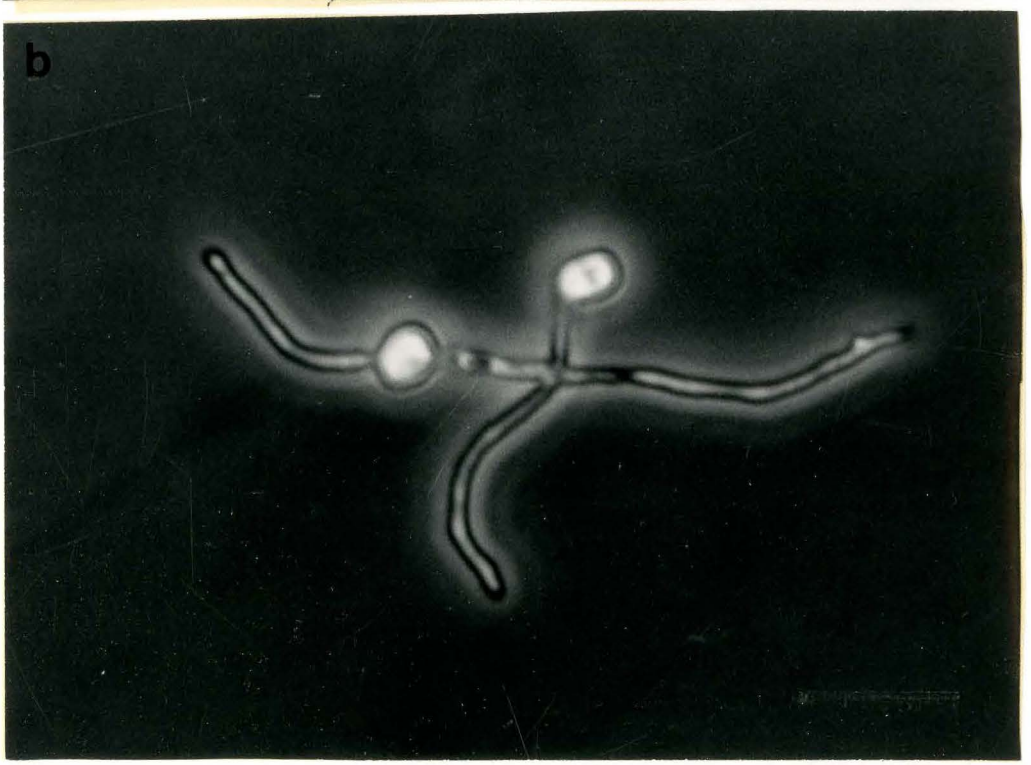
with multiple-buds which did not detach from the mother cell. As many as 12 cells could be seen attached together by the end of the 4 hour period in which they were observed (Fig. 13). The number of cells forming multiple-buds was also dependent on the pH and the concentration of glucose (Figs. 11 and 12). The lower the pH and the higher the concentration of glucose the more cells formed multiple-buds. At pH 3.7 approximately 60% of the cells induced by LAA formed multiple-buds when exposed to 200 mM glucose, while only 15% of those exposed to 5 mM glucose did so (Fig. 12). At pH 5.0 only 25% of the cells exposed to 200 mM glucose formed multiple-buds (Fig. 11). Cells induced by GlcNAc formed fewer multiple-buds than those induced by either LAA or proline. Only 39% of the cells induced by GlcNAc at pH 3.7 in the presence of 200 mM glucose formed multiple buds (data not shown).

Time of exposure of cells to glucose necessary for suppression of germ tube formation

Yeast cells were exposed to 200 mM glucose at various times following induction by proline to determine if the cells always retain their susceptibility to suppression by glucose. The cells rapidly lost their ability to have germ tube formation suppressed by glucose. When glucose was added 10 min after initiation of induction, 48% of the cells subsequently formed germ tubes, while 73% of the cells did so when glucose was added 30 min following induction. By 45 min, 91% of the cells were committed to hyphal growth and the further addition of glucose (up to 120 min) did not cause the hyphal cells to revert to budding during the subsequent 4 hr incubation period (data not shown). Furthermore, glucose suppressed germ tube formation only as long as it remained present in the medium. Yeast cells were preincubated for up to 4 h in

Fig. 13. Phase contrast micrographs of A) C. albicans yeast cells grown for 4 h in the presence of LAA and 200 mM glucose at pH 3.7, and B) C. albicans yeast cells induced to form germ tubes by LAA in the absence of glucose at pH 3.7 for 4 h. Bars, 10 μm. (This figure is from J. H. Pollack and T. Hashimoto, 1987, J. Gen. Microbiol. 133: 415-424.)


a


b

the salts solution buffered at pH 3.7, containing LAA and 200 mM glucose, before being washed by filtration. When the washed cells were reinoculated into the LAA induction solution, lacking glucose, at either pH 3.7 or 5.8, no less than 70% of the cells still formed germ tubes. When preincubated in the buffered salts and glucose only, for 4 h, 62% of the cells formed germ tubes when returned to the induction solution buffered at 5.8. Only if these cells were returned to the induction solution buffered at 3.7 was germ tube formation inhibited. However, even here, if these cells were preincubated for only 2 h, 62% of the cells formed germ tubes (Table 14). These results may have been affected by budding of the cells in the preincubation solution, and possible cell cycle changes.

Suppression of germ tube formation by other sugars

Some other sugars were also found to suppress germ tube formation at pH values below 5.8, but not above that value. These sugars, listed in Table 15, were galactose, mannose, fructose, xylose, and gluconic acid. Glucosamine inhibited germ tube formation, even when the pH was greater than 5.8, if its concentration was above 20 mM. However, at a concentration of 4 mM glucosamine, about 60% of the cells were induced to form germ tubes (data not shown). The non-metabolizable glucose analog, 3-0-methylglucose, also suppressed germ tube formation at pH 3.7. However, L-fucose, and L-rhamnose, which are also thought not to be metabolized by the cell, did not affect germ tube formation.

Suppression by glucose of other growth parameters at pH 5.8

Although glucose was shown not to affect germ tube formation at pH

Table 14. Relief of glucose suppression of germ tube formation following removal of glucose.

Time Glucose Removed (Min)	Cells Initially Exposed to Glucose Solution[1]		Cells Initially Exposed to Glucose in LAA[2]	
	Percent Cells Forming Germ Tubes When Returned to Induction Solution at pH:			
	3.7	5.8	3.7	5.8
0	92	100	92	100
15	87	93	96	98
30	86	98	90	98
45	82	95	91	98
60	83	96	79	95
90	ND[3]	92	96	93
120	62	68	82	87
150	ND	ND	64	88
180	33	77	66	81
240	4	62	69	73

1 Cells were incubated in glucose (200 mM), salts solution for the indicated time, after which they were washed by filtration and returned to LAA at the indicated pH. Values are the means of 2 to 3 independent experiments.

2 Cells were incubated in LAA containing glucose (200 mM) and salts for the indicated time, after which they were washed by filtration and returned to LAA at the indicated pH.

3 No data

Table 15. Suppression of germ tube formation by other sugars

Sugar (200 mM) added to induction solution[1,2,3]	Percent Cells Forming Germ Tubes at pH: 3.7	6.5
Galactose	27.3 ± 12.9	90.0 ± 9.6
Mannose	10.0 ± 1.7	97.0 ± 2.6
Fructose	22.0 ± 5.6	97.7 ± 2.5
Xylose	9.3 ± 5.5	79.3 ± 6.8
Gluconic Acid	7.3 ± 4.5	91.3 ± 7.5
Glucosamine	17.3 ± 3.2	7.8 ± 4.3
3-O-Methylglucose	5.6 ± 3.4	94.6 ± 3.4
L-Fucose (80 mM)	82.7 ± 3.2	93.0 ± 2.0
L-Rhamnose	94.6 ± 4.7	93.3 ± 4.9

1 Cells were induced with LAA to which 200 mM (except where indicated otherwise) of the sugars listed was added. The values are the means ± SD of 3 or more independent experiments. (This table is from J. H. Pollack and T. Hashimoto, 1987, J. Gen. Microbiol. 133: 415-424.)

b The following sugars tested were inhibitory either to growth or germ tube formation at both pH values: 2-deoxyglucose (1 mM), 2-deoxygalactose (10 mM), galactono-1,4-lactone, glucono-1,5-lactone, glucuronic acid, and L-sorbose.

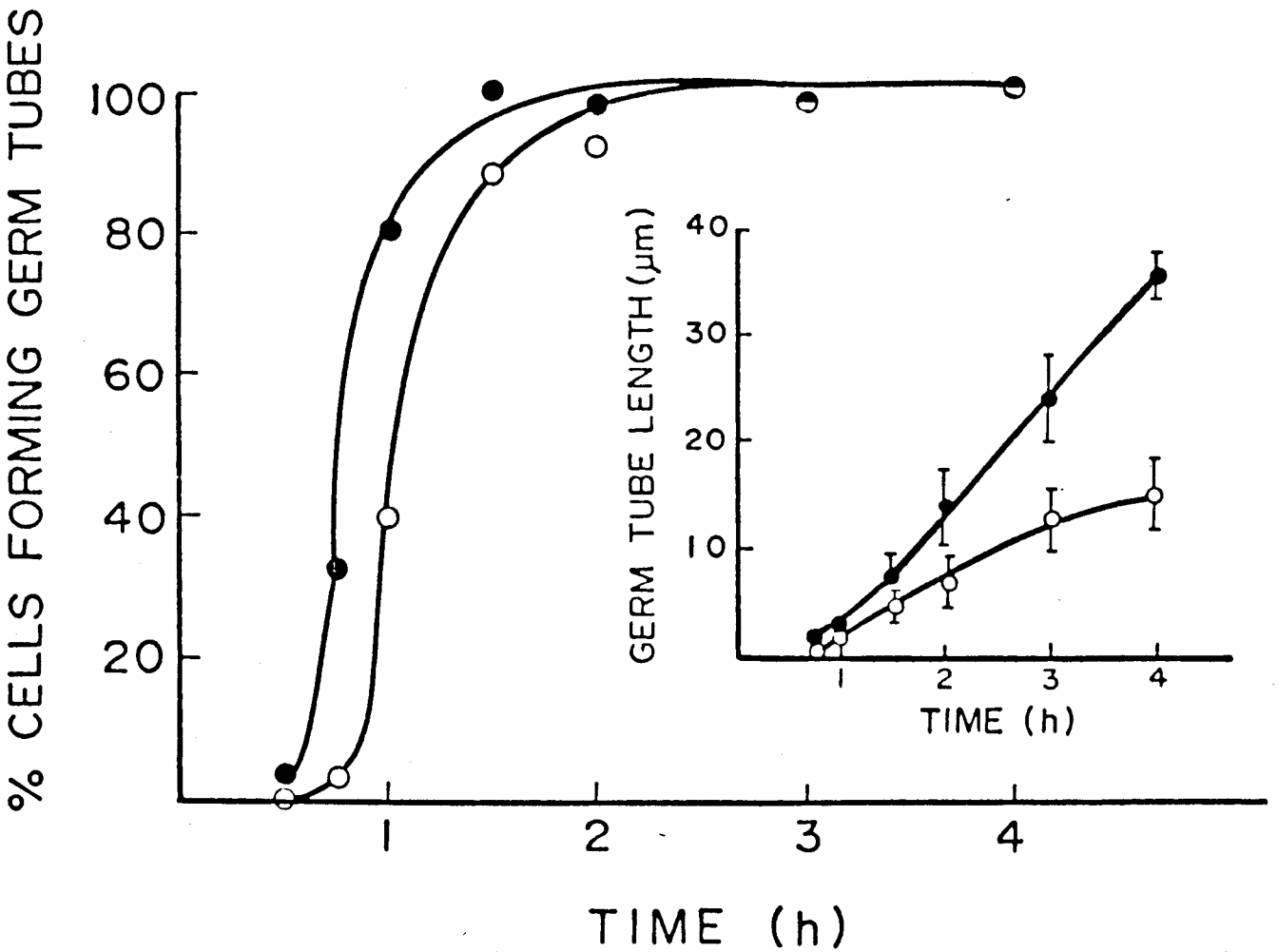
c The following sugars tested had no effect on germ tube formation at either pH value: glyceric acid, glycerol, erythritol, ribose, L-arabinose, D-arabinose, dulcitol, sorbitol, α-methylglucose, gulono-1,4-lactone, mannono-1,4-lactone, glucose-1-phosphate, glucosaminic acid, 6-phosphogluconic acid, glucoheptonic acid, mannoheptulose, cellobiose, lactose, melibiose, sucrose, trehalose, melezitose, and raffinose.

values above 5.8, we wished to determine whether glucose had any suppressive effects on growth at the higher pH values. We examined the kinetics of germ tube formation and germ tube length in the presence and absence of glucose at pH 5.8 (Fig. 14). In both instances glucose was stimulatory. In the presence of glucose, germ tube formation could be observed approximately 15 min earlier and the germ tube length after 4 h was more than twice that of those formed in the absence of glucose (35 μm versus 14 μm).

Effect of cyclic AMP on suppression of germ tube formation

Since the effect of glucose appeared to be similar to that of catabolite repression in bacteria, we examined whether cAMP or theophylline had any effect on suppression of germ tube formation. Neither dibutryl cAMP (4 mM) or theophylline (5 mM) had any effect on germ tube formation in the presence or absence of 200 mM glucose (data not shown).

Fig. 14. Kinetics of germ tube formation in the presence and absence of glucose at pH 5.8. C. albicans yeast cells were induced to form germ tubes at pH 5.8 and 37°C by 4 mM proline in buffered salts solution in the presence (-●-) or absence (-O-) of 200 mM glucose. The average length of 50 germ tubes formed at each interval is indicated in the insert. Values are the result of a representative experiment. Bars represent SD. (This figure is from J. H. Pollack and T. Hashimoto, 1987, J. Gen. Microbiol. 133: 415-424.)


% CELLS FORMING GERM TUBES
100
80
60
40
20
1
2
3
4
TIME (h)
GERM TUBE LENGTH (μm)
40
30
20
10
1
2
3
4
TIME (h)

DISCUSSION

Both glucose and pH individually have been reported to influence the ability of C. albicans to form germ tubes. To our knowledge, this is the first report which demonstrates a relationship between the effects of pH and glucose.

The effect of glucose on germ tube formation in C. albicans has been a matter of dispute. Nickerson and Mankowski (1953) first reported that glucose inhibited filamentation in C. albicans. Widra (1964) reported that cells grown on neopeptone agar containing potassium phosphate, but lacking glucose, formed mycelia, while those grown on the same agar containing 0.25% glucose grew in the yeast phase. Dabrowa et al. (1976) found that glucose concentrations as low as 1 mM suppressed germ tube formation induced by proline at pH 7.0. Odds et al. (1978) reported that while peptones containing 0.2% glucose were equally effective in inducing germ tubes, Mycophil broth, which contained a preformulated glucose concentration of 4%, did not support germ tube production above the control levels. Hrmova & Drobnica (1981) observed that exposure to an initial glucose concentration greater than 10 mM resulted in suppression of germ tube formation at 28°C, at pH 6.5, in cells induced by the combination of glucose and $(NH_4)_2SO_4$. Nolting et al. (1982) also reported that glucose concentrations above 1 mg ml^{-1} repressed germ tube formation. Conversely, Nishioka & Silva-Hutner (1974) found that 2% glucose did not suppress germ tubes induced by glutamate while Evans et al. (1975b) found that even 20% glucose had no effect on germ tube formation induced by peptone at pH 7.4. Barlow et al. (1974) also reported that glucose (10 mM) was required to maintain filamentation induced by serum, and Land

et al. (1975) reported that 1 M glucose was necessary to obtain moderate filamentation in the presence of NH_4Cl.

Similarly, differences have been reported as to the effect of pH on germ tube formation. Most workers have reported a pH optimum for germ tube formation which varies from approximately 6.6 to 7.4 (Evans et al., 1975b; Simonetti et al., 1974; Buffo et al., 1984). There are only a few reports of germ tube formation occurring below pH 6.0 (McClary, 1952; Shepherd & Sullivan, 1976) and only one previous report of germ tube formation occurring as low as pH 3.0 (Pollack & Hashimoto, 1985). This is despite the fact that C. albicans is known to grow at very low pH values (Odds & Abbott, 1980) and that the pH of the human vagina, a common site for Candida infections, is between 4 and 5 (Ryley, 1986).

The results presented in this chapter suggest that the dearth of reports showing the ability of C. albicans to form germ tubes at low pH values, may be due, in part, to the presence of glucose in the induction solutions used by many groups. Unlike the solutions used here, many induction solutions are poorly buffered, so that even if the initial pH would have allowed germ tube formation to occur in the presence of glucose, a quick drop in pH could suppress germ tube formation. In fact, just increasing the pH of a non-inducing solution, such as Sabouraud dextrose broth to 7.5, was found sufficient to allow germ tube formation to occur (Evans et al., 1974). We also found that buffering SDB at pH 5.8 resulted in germ tube formation (data not shown). This is not to say that other factors do not affect the ability of C. albicans to form germ tubes at non-optimal pH values. Shepherd et al. (1980) showed that the buffer used may also affect the ability of the cells to form germ tubes. We have found that the concentration of Na^+ created by

adjusting the pH of some buffers, such as tris-maleate, with NaOH, may also inhibit germ tube formation under certain conditions. Thus, we found that few germ tubes were formed above pH 7.5 when cells were induced by a low concentration of proline or GlcNAc (0.08 mM) if tris-maleate was used as the buffer, but almost all cells produced germ tubes if HEPES or TAPS were used (data not shown). Others have also suggested that inorganic phosphate affects the ability of C. albicans to form germ tubes (Widra, 1964; Dabrowa et al., 1976; Odds et al., 1978).

The results presented here do not agree with the recently proposed pH-regulation of germ tube formation in C. albicans (Mitchell & Soll, 1979; Buffo et al., 1984). The proposal of Soll and co-workers is based on the assumption that germ tube or bud formation can be controlled solely by regulating the pH of the inducing solution, similar to the more common temperature or nutritional regulation of germ tube formation. Their proposal is tempered by the statement that there are variant strains which are capable of forming germ tubes at pH values below 4.0 and that their transition points are "rigidly defined only for the environmental conditions and growth history of the cells" induced under their experimental regimen (Buffo et al., 1984). However, we were able to reproduce their results with different strains and under different growth conditions. Since we were able to obtain germ tubes at pH values as low as 3.0 only by removing glucose from any number of induction media, we believe that pH cannot be considered the sole determinant of germ tube formation. At most, pH can be considered an "indirect" regulator or co-regulator of dimorphism in C. albicans.

When grown in the presence of glucose at low pH values, the budding cells, also, apparently did not separate, which resulted in the formation of

large clusters of multiple-budded cells or pseudohyphae. Similar modes of growth have been reported previously in response to glucose at lower temperatures (Soll & Bedell, 1978; Dujardin et al., 1980). It might be useful to use this phenomenon to distinguish between growing and non-growing cells at low pH or low temperatures. These results also may indicate that the mechanism of glucose suppression at low pH is by stimulating budding, rather than by inhibiting germ tube formation.

The results presented here also differ from those of others with respect to commitment to germ tube formation. We found that by 45 min germ tube formation could no longer be suppressed by the addition of glucose, while in most situations tested, removal of glucose resulted in cessation of suppression. It would seem, therefore, that C. albicans is committed to hyphal formation very early, but is never committed to the budding form of growth. The time for commitment to germ tube formation compares favorably with those times reported by Evans et al. (1975a) and Chaffin & Wheeler (1981). However, those studies and those of Mitchell & Soll (1979), also found that at some point the cells were committed to budding. Besides using different criteria (temperature or pH shift) to determine commitment, it is possible that the previously mentioned experimenters did not allow sufficient time following switching the growth conditions, to properly assess the pluripotency of the cells to form germ tubes. The cells in the present study were incubated for 4 h post-transfer while cells used by both Mitchell & Soll (1979) and Chaffin & Wheeler (1981), were incubated for a total of only 4 h, including the time prior to the switch in growth conditions. Furthermore, the previous authors claimed that C. albicans yeast cells must be in the stationary phase in order for germ tube formation to proceed. This claim has been

questioned by others (Mattia & Cassone 1979; Ahrens et al., 1983; Soll & Herman, 1983). However, it is possible that the extensive washing that the cells received prior to their transfer to the new growth conditions, was equivalent to the starvation period which Soll & Herman (1983) found sufficient to activate the cells, although it was for a much shorter period (4 to 5 min).

Suppression of differentiation by glucose in other organisms has been reported previously. Bartnicki-Garcia (1968) reported that glucose inhibited initiation of filamentation, but not hyphal elongation, in Mucor rouxii. Redshaw et al. (1976) observed that some Streptomyces species were inhibited from forming aerial mycelia in the presence of glucose only when the pH was between 5 and 5.5, but not when the pH value was higher. These reports, and those on glucose repression in C. albicans, suggest that the action of glucose is mediated by catabolite repression or inactivation. The data presented here is inconclusive in regards to whether a catabolic product of glucose is mediating the effect since in addition to the other sugars, a supposedly non-metabolizable analog of glucose also suppressed germ tube formation. However, cAMP did not seem to mediate the effect. This is not what was expected since cAMP has been reported to affect dimorphism in C. albicans (Niimi et al., 1980; Chattaway et al., 1981). On the other hand, it is not clear what role cAMP plays in catabolite repression in yeasts (Eraso & Gancedo, 1984, 1985). The actual mode of action of glucose remains to be determined. Some interesting possibilities suggested by others are increasing the rate of proteolytic degradation of the nutrient inducer transport system (Horák & Řihová, 1982), the suppression of production of some key enzyme such as chitinase (Monreal & Reese, 1969), or the repression of the catabolic pathway for the inducer (Singh & Datta, 1978).

CHAPTER V

FORMATION OF GERM TUBES BY CANDIDA ALBICANS AT SUBOPTIMAL TEMPERATURES

ABSTRACT

Candida albicans formed germ tubes at temperatures between 25° and 30°C in solution when incubated without shaking, in the presence of bicarbonate (2 mg ml^{-1}). Other conditions depended on the inducer used. Proline could induce germ tube formation optimally only when its concentration was between 200 and 400 mM. The addition of 0.05 to 5 mM glucose to a 5 mM proline induction solution, though, did allow germ tube formation at 30°C but not at 25°C. A concentration of 0.05 mM N-acetylglucosamine (GlcNAc) was sufficient to induce germ tube formation. GlcNAc could induce germ tube formation at 30°C but not at 25°C. GlcNAc-induced germ tube formation was most reproducible when the cells were first incubated in water for 16 to 24 h at 20°C (priming). No germ tubes were formed by any inducer at 20°C or below. Germ tubes induced by proline could be formed at pH values between 3.8 and 9.0 at 30°C, but only between 7.0 and 7.5 at 25°C. Glucose (400 mM) did not suppress germ tube formation at 30°C but only 5 mM was sufficient to cause a 65% suppression at 25°C.

INTRODUCTION

It has been generally accepted that three factors regulate germ tube formation in Candida albicans: temperature, pH, and the presence of a suitable metabolizable inducer. Recently, however, the requirement for each of these factors has been questioned. Sims (1986) has shown that a 10% CO_2 atmosphere could replace the requirement for a 37°C incubation temperature and allow germ tube formation to occur at 25°C. Shepherd & Sullivan (1982) have demonstrated that the inducer need not be metabolized for germ tube formation to occur. We have shown that removal of glucose from the induction solution allows germ tube formation to proceed at pH values as low as 3.0 (Pollack & Hashimoto, 1987). Having established that germ tube formation is not regulated by pH alone, but rather by a critical balance between pH and the presence of glucose, we wished to determine if carbon dioxide is the only factor which moderates the affect of temperature on germ tube formation. We found that the temperature regulation is affected by many factors including concentration of the inducer, the presence of glucose, pH, and the growth state of the cells. In addition, bicarbonate could replace carbon dioxide for germ tube formation to occur at 30°C or lower.

METHODS

Growth and preparation of cells. Candida albicans (ATCC 58716) was grown on Sabouraud dextrose agar at 37° for 24 h. The yeast cells were washed by vacuum filtration 10 times being careful to resuspend the cells between each washing. If the cells were used immediately, they were termed non-primed cells. In a number of circumstances the optimal number of germ tubes could be obtained only if the cells were first incubated overnight in water at 20°C. These cells were termed primed cells.

Conditions for induction of germ tubes. Both primed and non-primed cells (final concentration of 7.5 x 10^5 cells ml^{-1}) were inoculated into a buffered, salts solution (final volume, 1.0 ml, in a 12 X 75 mm glass tube) containing either L-proline (4 mM at 37°C, 350 mM at 30°C or below) or N-acetyl-D-glucosamine (GlcNAc)(4 mM at 37°C, 50 mM at 30°C) as inducers. The salts used were: $FeSO_4$ (0.1 mg L^{-1}), KCl (400 mg L^{-1}), $MgSO_4.H_2O$ (200 mg L^{-1}), $NaH_2PO_4.H_2O$ (125 mg L^{-1}), NaCl (6400 mg L^{-1}), and $NaHCO_3$ (2000 mg L^{-1}). Unless otherwise indicated, the buffer used was either Tris(tris[hydroxymethyl]amino methane)-maleate or HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) adjusted to a pH between 7.0 and 7.2. In order to test the effect of pH on germ tube induction the following buffers were used: sodium citrate-sodium phosphate (pH 2.5 to 6.5), HEPES (pH 7.0 to 8.0), MES (2-[N-morpholino]ethanesulfonic acid) (pH 5.5 to 6.5), sodium succinate (pH 4.0 to 5.0), Tris-maleate (pH 5.0 to 8.0), sodium trans-aconitate (2.5 to 6.0), or TAPS (tris[hydroxymethyl]methylaminopropanesulfonic acid)(pH 7.5 to 9.5). With the exception of citrate-phosphate buffer, all buffers were adjusted to

the desired pH with NaOH. The concentration of the buffer salt was 0.05 M. In some experiments glucose was added to the induction solution. The cells were incubated at atmospheric conditions when the temperature used was either 37° or 30°C. In the initial experiments to obtain germ tubes at 25°C the atmospheric conditions were altered by evacuating a vacuum desiccator jar attached to a mercury manometer (Fisher Scientific) using a house vacuum line and then admitting either carbon dioxide or nitrogen into the jar until there was an decrease of 10% in the level of mercury. The jar was then filled with air, until the mercury level almost reached zero, but still leaving a slight negative pressure. This resulted in a 2% decrease in the concentration of oxygen. In later experiments, it became clear that if the volume of the induction solution was 1 ml or greater (the height of the solution was 1.5 mm), and the cells were incubated without shaking, there was no need to resort to changing the atmospheric conditions. After 4 to 5 h incubation, the cells were examined for germ tubes using a phase contrast microscope, as described previously (Pollack & Hashimoto, 1987).

RESULTS

The effect of concentration of the inducer on germ tube formation

The first important factor affecting germ tube formation at temperatures below 37°C, was the concentration of the inducer. At 37°C both proline and GlcNAc could induce germ tube formation in more than 80% of the cells at a concentration as low as 0.04 mM (Table 16). However, 100 mM and 200 mM proline was required at 30°C and 25°C, respectively, in order to for the maximal number of cells to form germ tubes (Figure 15). At these concentrations approximately 80% of the cells at 30°C and 70% of the cells at 25°C formed germ tubes. The germ tubes formed at 25°C were true hyphae, as long as 40 μm long, without constrictions at their base (Fig. 16). The high concentration of proline required for germ tube formation at these temperatures made us suspect that germ tubes might possibly be induced by a non-specific action caused by the high osmolarity of the solution. We therefore tested a number of other non-inducing amino acids and analogs of proline to see if they were able to induce formation of germ tubes at high concentrations. None of the following compounds were capable of inducing germ tube formation at concentrations up to 500 mM (where possible): glycine, lysine, serine, hydroxyproline, α-amino-butyric acid, δ-amino-valeric acid, picolinic acid, pyrrolidine, and pyrrole. However, if 0.05 to 5 mM glucose was added to the induction solution, germ tubes were formed by 5 mM proline at 30°C, but not at 25°C (data not shown).

Requirements for priming of cells prior to induction of germ tubes

Unlike proline, even 0.05 mM GlcNAc induced 40% of the cells to form

Table 16. Effect of concentration of inducer on germ tube formation at 37°C.

Concentration of inducer	Percentage of cells forming germ tubes when induced by:[1]	
	Proline	GlcNAc
0.1	93.7 ± 5.7	93.7 ± 5.5
0.04	83.0 ± 8.8	80.8 ± 7.1
0.01	38.0 ± 2.8	27.3 ± 8.1
0.004	18.0 ± 4.2	12.0 ± 6.1

1 Values are the means ± SD of 3 or more independent experiments.

Fig. 15. Effect of temperature on the concentration of proline required to induce germ tube formation in C. albicans. Cells were incubated at the indicated concentrations of proline in buffered salts solution at either 30°C (-0-) or 25°C (-●-), for 5 h. Values represent the average of 3 or more independent experiments. Bars represent SD.

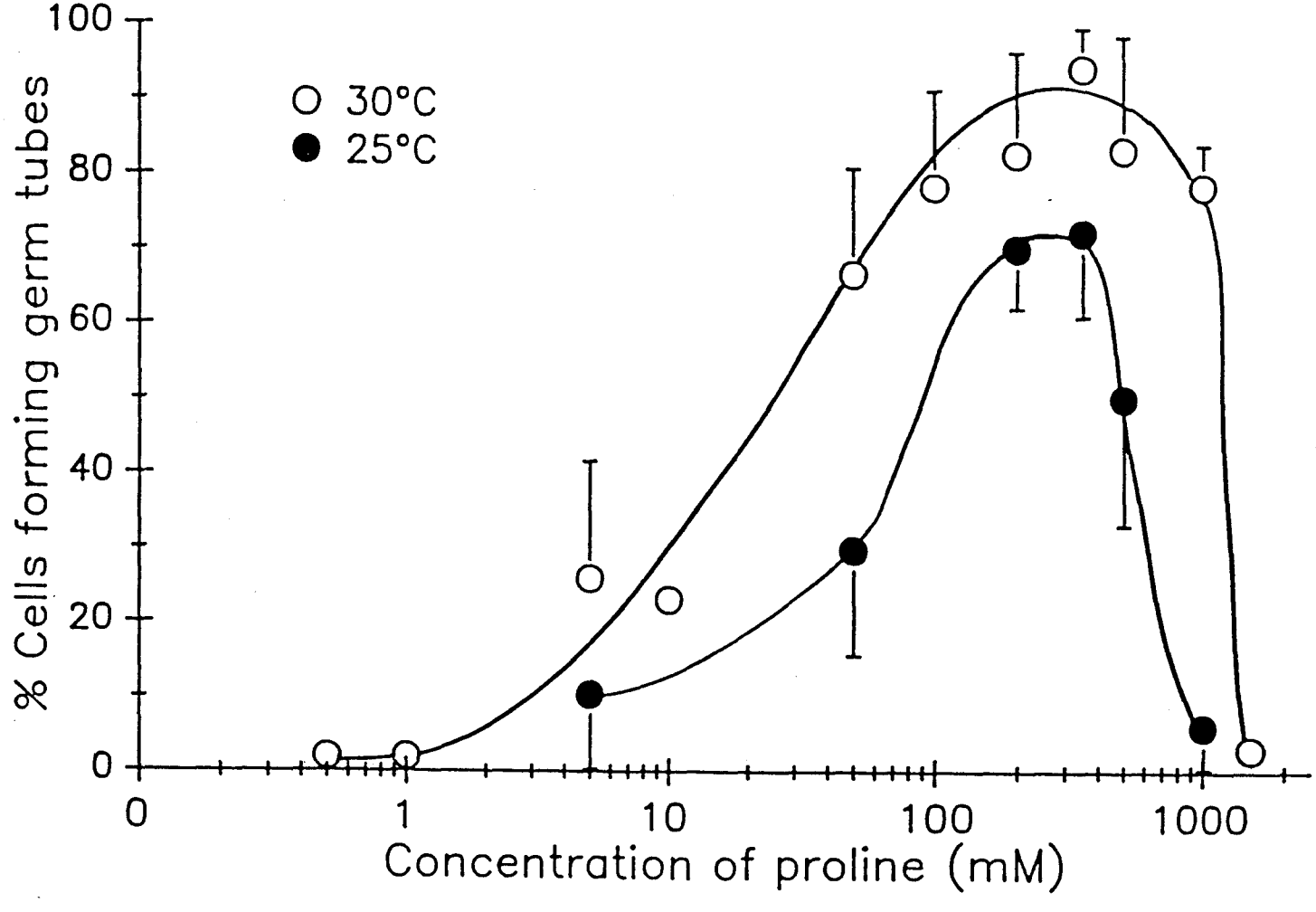
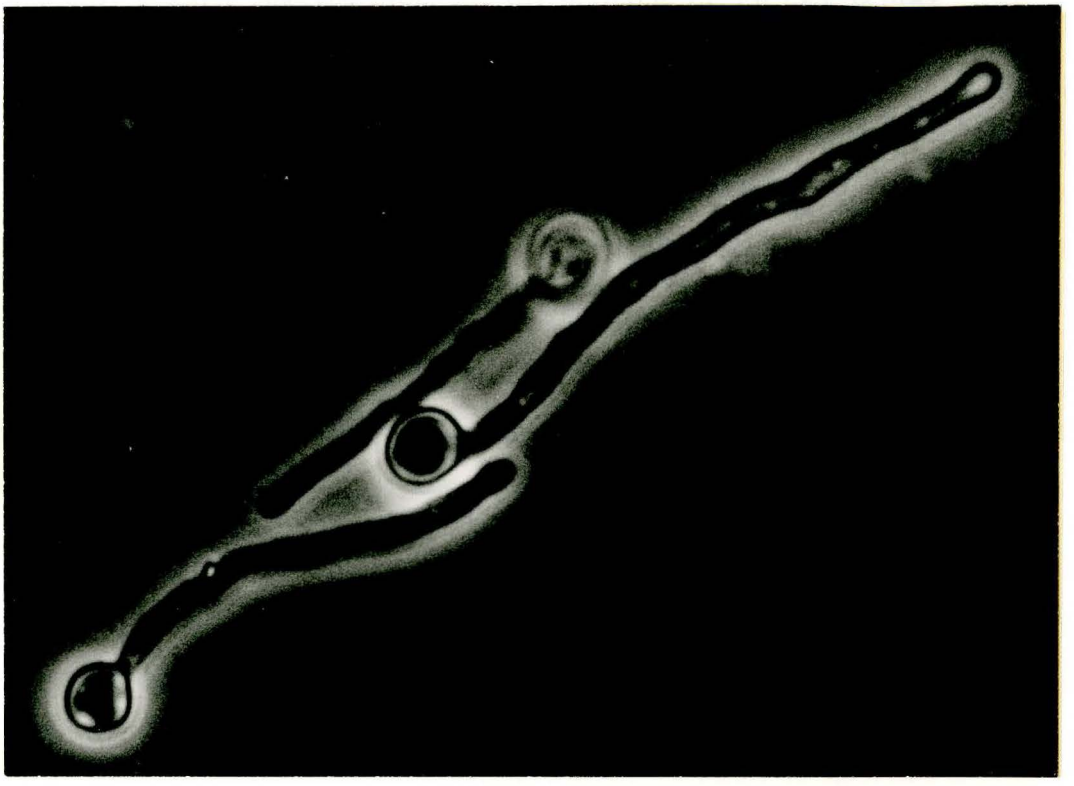

100
80
60
40
20
0
% Cells forming germ tubes
30°C
25°C
0
1
10
100
1000
Concentration of proline (mM)

Fig. 16. Phase contrast micrograph of C. albicans induced to form germ tubes by proline (350 mM) in buffered salts solution at 25°C. Note the long tubes, without constrictions at the base or along their length. Bar, 10 μm.



germ tubes at 30°C, a concentration not much greater than the minimum needed at 37°C (Fig. 17, Table 16). Between 80 and 90% of the cells formed germ tubes when induced by 0.5 to 200 mM GlcNAc. However, in order to elicit such a response, consistently, the GlcNAc-induced cells had to first be primed (Figure 17). Non-primed cells formed approximately one-fifth less germ tubes than primed cells formed. This effect was magnified if the pH was only slightly changed from the pH optimum of 7.0 (See below). Increasing the concentration of GlcNAc did not significantly increase the number of germ tubes formed by non-primed cells. It was not necessary to prime cells induced with proline to obtain germ tubes at any temperature tested.

Minimum temperature allowing germ tube formation

The minimum temperature at which germ tube formed depended on the inducer. We were not able to obtain germ tubes at 25°C with cells induced with GlcNAc. Likewise, no more than 20% of the cells induced by ethanol formed germ tubes at 30°C, and none below 28°C (Pollack & Hashimoto, 1985). Approximately 80% of non-primed cells induced with the combination of ammonium chloride (20 mM) and glucose (1 mM) also formed germ tubes at 30°C, but not at 25°C, although only 0.05 mM of each was required at 37°C. Approximately 20% of the proline-induced cells formed germ tubes at 22°C. We found no conditions capable of supporting germ tube formation at 20°C or below.

Requirement of bicarbonate for germ tube formation.

Table 17 illustrates the absolute requirement for bicarbonate in order to obtain germ tubes at 30°C or below, irrespective of whether proline or

Fig 17. Effect of concentration of GlcNAc on germ tube formation at suboptimal temperatures. Primed (- 0 - , - □ -) and non-primed (- ● -) cells were incubated at the indicated concentration of GlcNAc in buffered salts containing 2 mg ml^{-1} at either 30°C (-0-, - ● -) or 25°C (- □ -), for 5 h. Values represent the average of 3 or more independent experiments. Bars represent SD.

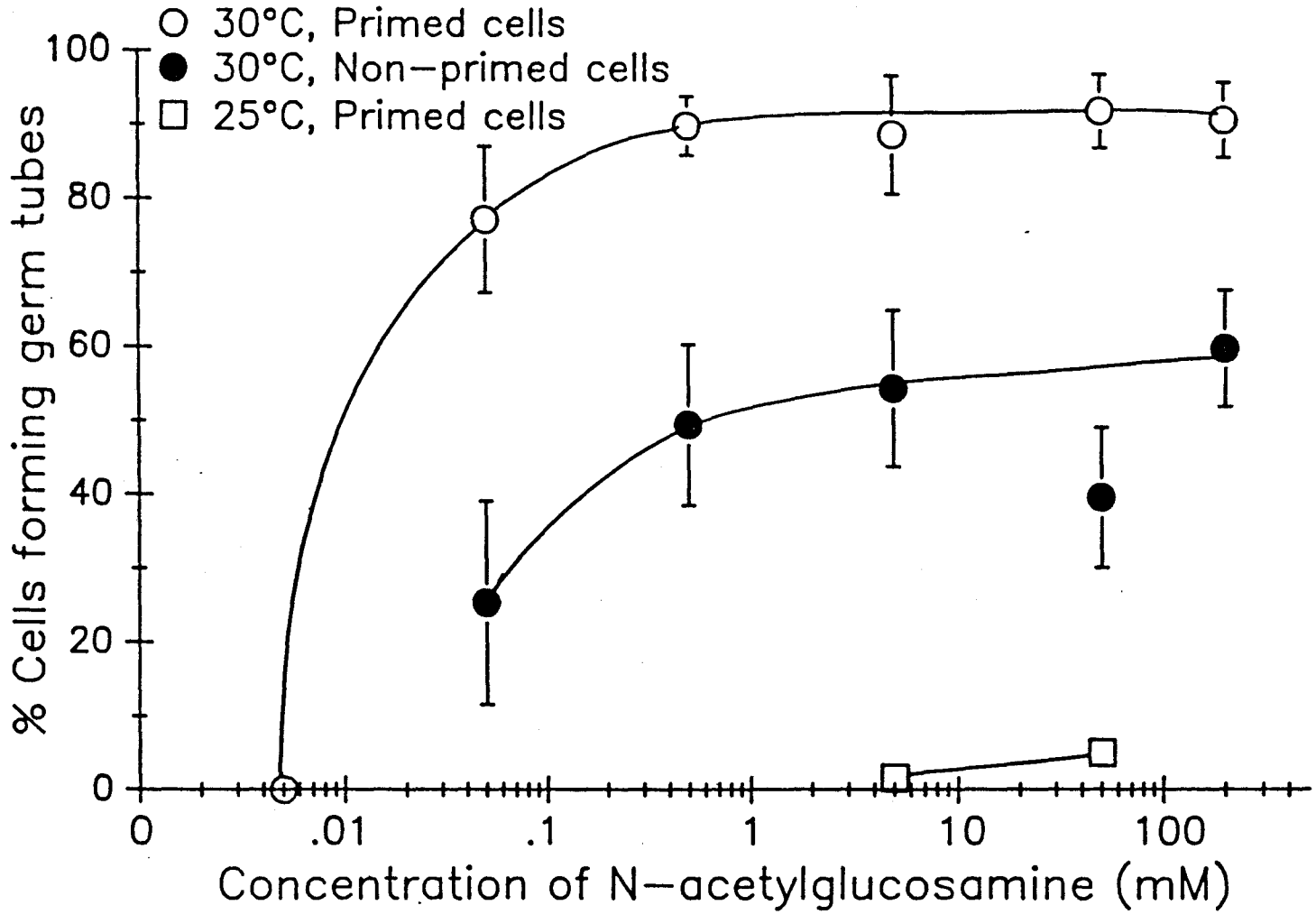

30°C, Primed cells
30°C, Non−primed cells
25°C, Primed cells
% Cells forming germ tubes
100
80
60
40
20
0
0
.01
.1
1
10
100
Concentration of N−acetylglucosamine (mM)

Table 17. Effect of Bicarbonate on germ tube formation

	Percentage of cells forming germ tubes[1]				
	Induction conditions				
Concentration sodium bicarbonate (mg/ml)	Proline (350 mM) 30°C pH 5.0	Proline (350 mM) 30°C pH 6.2	Proline (350 mM) 30°C pH 7.0	GlcNAc (50 mM) 30°C pH 7.0	Proline (350 mM) 25°C pH 7.0
0	0	8 (10)[2]	21 (56)	3	1
0.1	19	16	54	20	0
0.25	43	44 (88)	83 (86)	30	12
0.5	65	70 (88)	95 (98)	43	29
1.0	86	89 (93)	99	65	41
2.0	96	91	97	78 (95)	75

1 Values are the means of three or more independent experiments using non-primed cells.

2 Values in parentheses were obtained using primed cells.

GlcNAc were used as inducers. Whereas the absence of bicarbonate from cells induced at 37°C had little effect, its absence from the solutions used to induce cells at 30°C resulted in greatly reduced germ tube formation, and in essentially no germ tube formation at 25°C. The amount of bicarbonate needed seemed to be dependent both on the pH and on whether the cells were primed. When primed, about 90% of the cells induced by proline at pH 6.2 formed germ tubes when provided with 0.25 mg ml^{-1}. However, only 44% of the non-primed cells formed germ tubes when supplied with the same concentration of bicarbonate. At the optimum pH of 7.0 priming allowed 50% of the cells to form germ tubes without the addition of exogenous bicarbonate. The addition of 2 mg ml^{-1} bicarbonate was sufficient under all conditions tested to obtain optimal percentage of cells forming germ tubes. Doubling the concentration to 4 mg ml^{-1} was without effect. However increasing the concentration to 8 mg ml^{-1} was in general inhibitory (data not shown).

Influence of pH on germ tube formation at low temperatures

pH was the next factor affecting germ tube formation at low temperature which was considered. Nearly 100% of the cells induced with 350 mM proline formed germ tubes at 30°C over the same wide range of pH values (3.8 to 9.0) as were obtained at 37°C when induced with 4 mM proline (Pollack & Hashimoto, 1987). At 25°C, however, germ tube formation was limited to the narrow pH range of 7 to 7.5 (Fig. 18). Non-primed cells induced by GlcNAc at 30°C were also restricted to the same narrow pH range. However, primed cells induced by GlcNAc were able to form germ tubes over a much wider range (Fig. 19).

Fig. 18. Effect of pH on proline-induced germ tube formation in C. albicans at suboptimal temperatures. Cells were incubated in buffered salts containing 350 mM proline and 2 mg ml^{-1} sodium bicarbonate for 5 h at either 30°C (-0-) or 25°C (-●-). The buffers used to control the pH are listed in the Methods section. Values represent the average of 3 or more independent experiments. Bars represent SD.

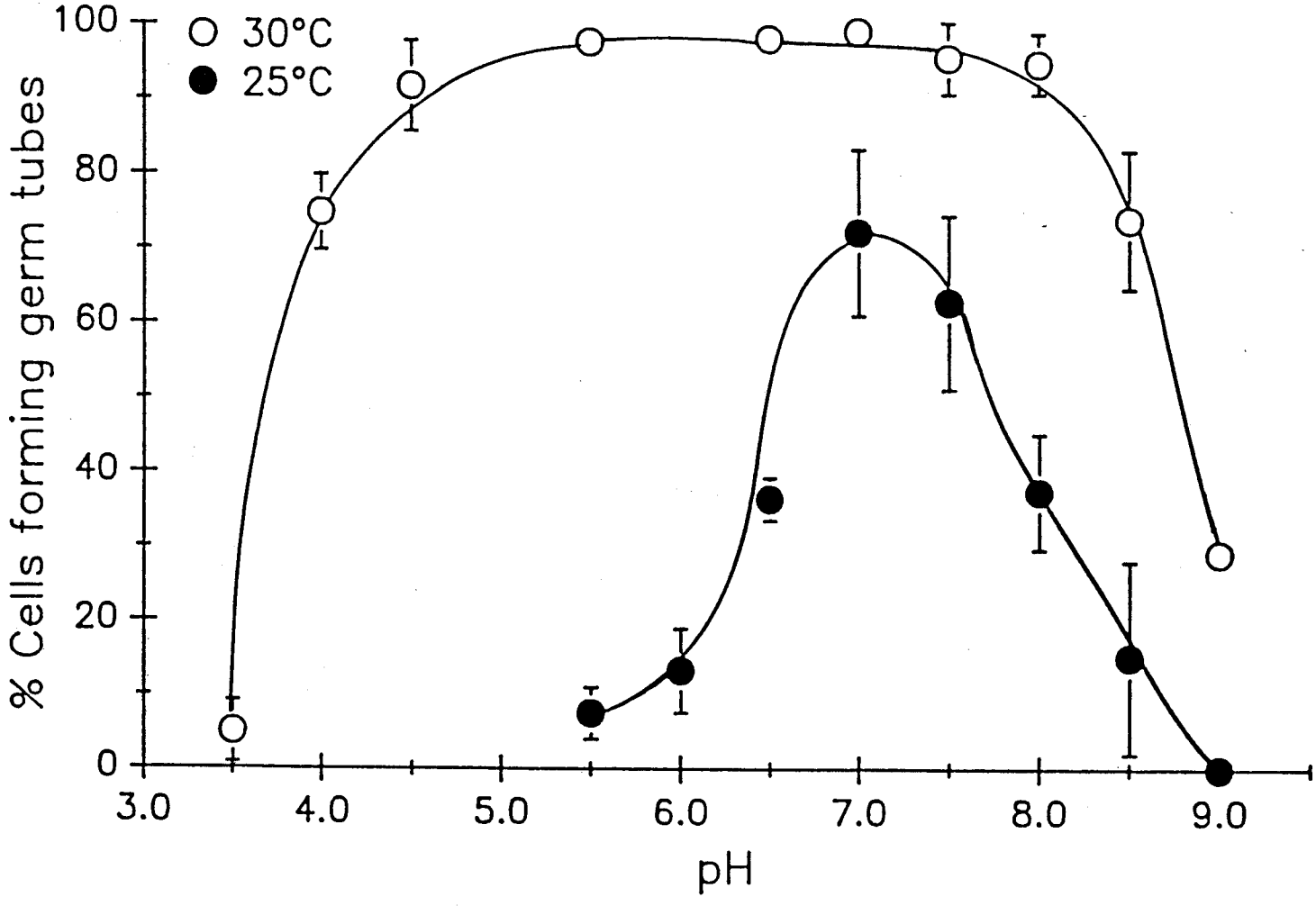
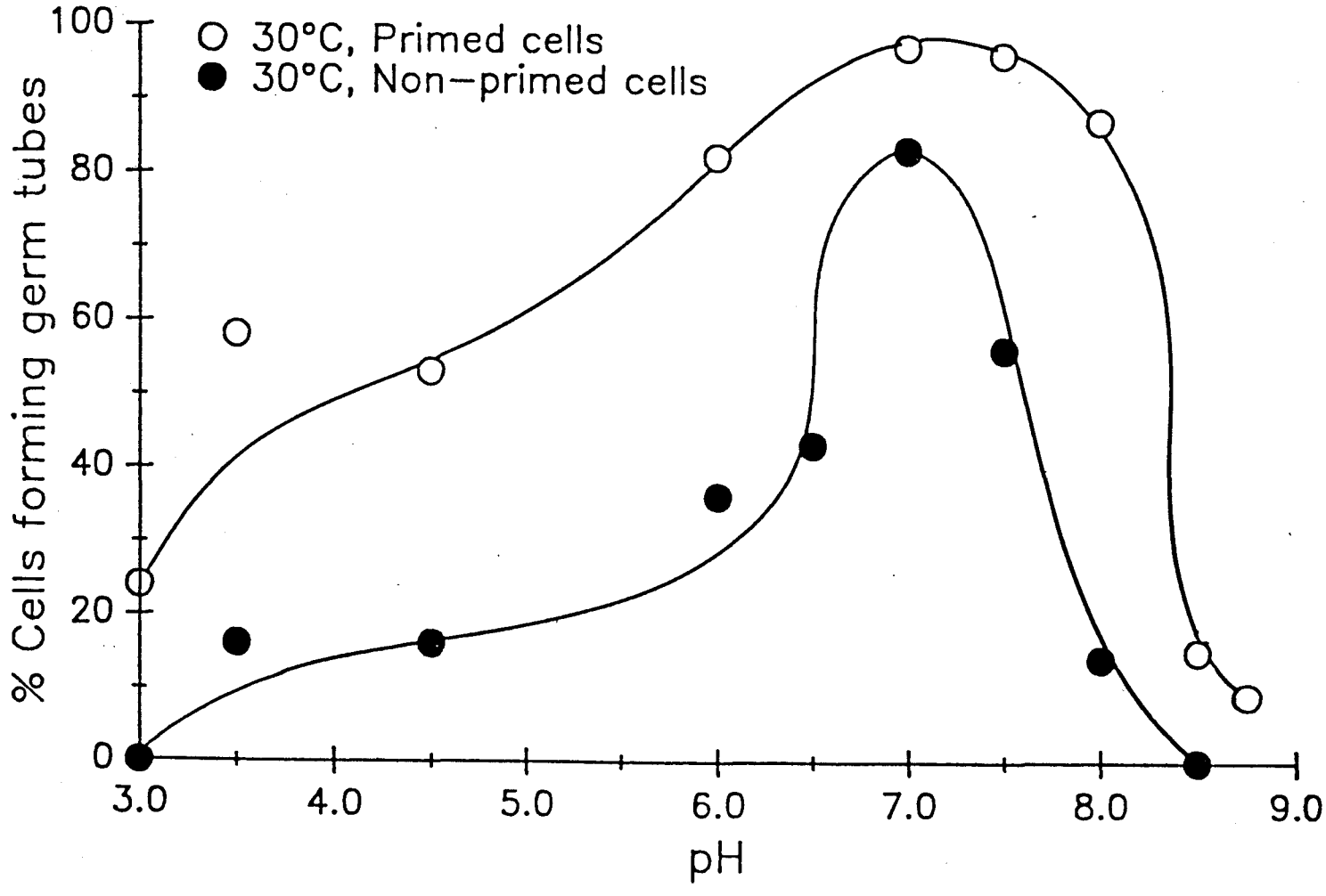

100
80
60
40
20
0
% Cells forming germ tubes
30°C
25°C
3.0
4.0
5.0
6.0
7.0
8.0
9.0
pH

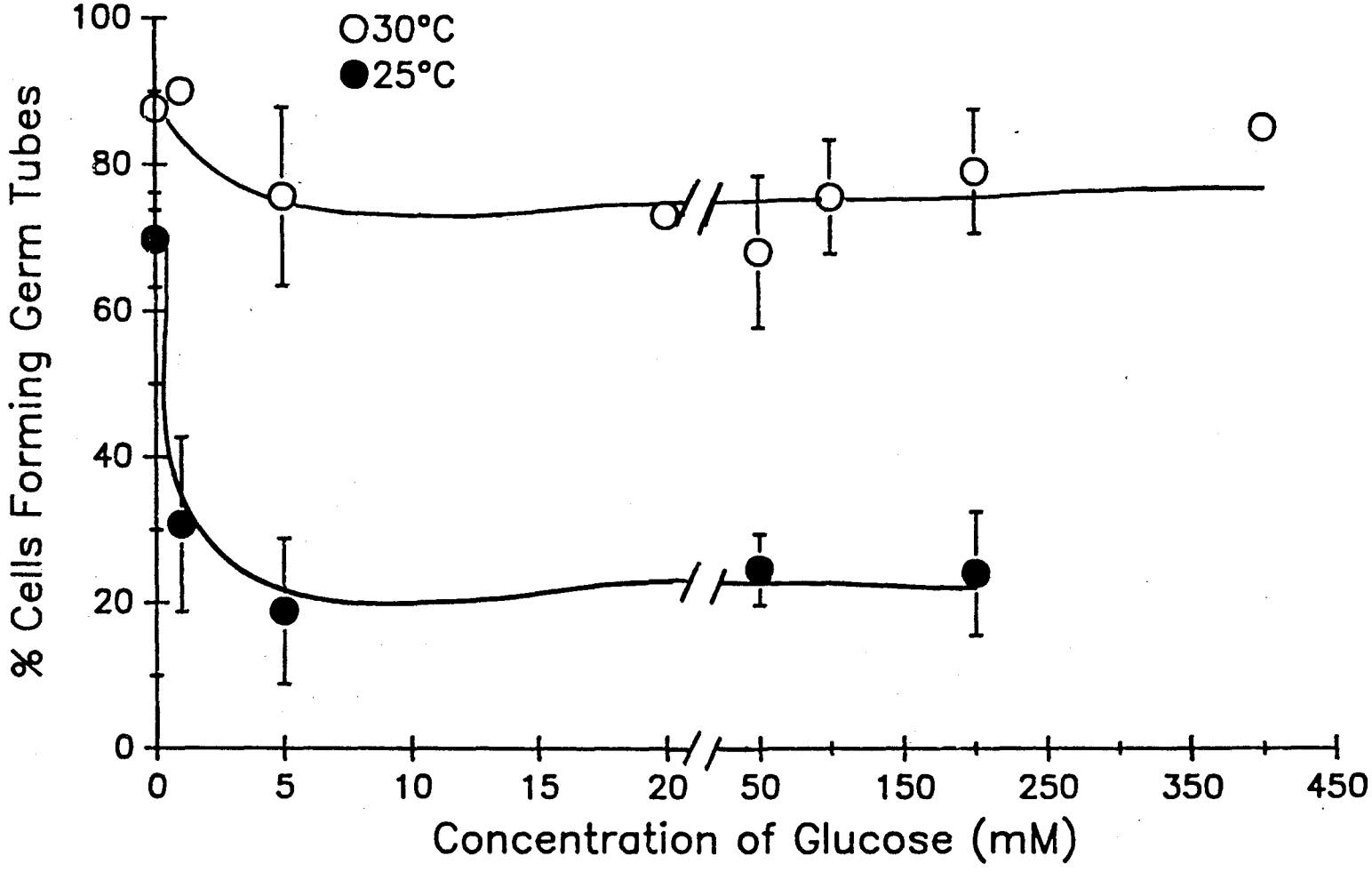
Fig. 19. Effect of pH on GlcNAc-induced germ tube formation at 30°C. Primed (- ○ -) and non-primed (- ● -) cells were incubated in buffered salts containing 50 mM GlcNAc and 2 mg ml^{-1} at 30°C. The buffers used to control the pH are listed in the Methods section. Values are the means of 2 experiments.


100
80
60
40
20
0
% Cells forming germ tubes
30°C, Primed cells
30°C, Non–primed cells
3.0
4.0
5.0
6.0
7.0
8.0
9.0
pH

Effect of glucose on germ tube formation at low temperatures

Glucose also inhibited germ tube formation, but only at 25°C. At 30°C it was almost without any effect, even at 400 mM glucose. As little as 1 mM glucose was sufficient to reduce germ tube formation by more than half, while 5 mM glucose suppressed germ tube formation in approximately 65% of the cells which formed germ tubes in the absence of glucose. Additional increases in the concentration of glucose did not further suppress germ tube formation (Fig. 20).

Fig. 20. Influence of the concentration of glucose on proline-induced germ tube formation at suboptimal temperatures. Cells were incubated for 5 h at either 30°C (-0-) or 25°C (-●-) in buffered salts containing 350 mM proline, 2 mg ml^{-1}, and the indicated amount of glucose. Values represent the mean of at least 3 experiments. Bars represent SD.


O30°C
●25°C
% Cells Forming Germ Tubes
100
80
60
40
20
0
0
5
10
15
20
50
150
250
350
450
Concentration of Glucose (mM)

DISCUSSION

Many of the published reports on the factors affecting germ tube formation in C. albicans are contradictory, particularly those dealing with the roles of glucose and temperature. In general, these reports have maintained that germ tube formation is limited to rather restricted and defined conditions. Differences between the requirements for germ tube formation found in previous studies were usually attributed to the use of different strains rather than to differences in media and growth conditions. It is only recently that Sevilla & Odds (1986) have emphasized the importance of arriving at a model for germ tube formation only after first ascertaining the "consistency of (the) observations in different hypha-stimulating environments." We have also stressed the importance of using the simplest media to determine which factors affect germ tube formation, in order to minimize possible inconsistencies due to the presence of extraneous compounds. Using that approach we demonstrated that pH alone did not regulate germ tube formation, as had been suggested by others (Mitchell & Soll, 1979; Buffo et al., 1984), but only in conjunction with glucose (Pollack & Hashimoto, 1987).

A temperature of 37°C, too, has been considered an absolute requirement for germ tube formation in liquid media (Dabrowa & Howard, 1983; Odds, 1985). While it is common to find some hyphae on solid media incubated at 25°C for 18 to 24 h, abundant hypha are not formed unless the agar is either scratched using the "Dalmau" inoculation technique, or the agar covered with a glass coverslip or both (Dalmau, 1929; Bentham, 1932; Wickerham & Rettger, 1939). It has long been thought that reduced oxygen tension was involved in the production of the hyphae formed under the coverslip, and also the reason

that hyphae are more common in liquid (at 37°C) rather than solid media (Wickerham & Rettger, 1939; McClary, 1952). Sims (1986), however, attributed hyphal formation to increased concentration of CO_2, either physically added to the system, or accumulated metabolically, a conclusion also supported by Langeron & Talice (1932) and Mardon et al. (1969). Sims' investigation also showed that an induction temperature of 37°C is, in most cases, required only in the absence of carbon dioxide. In the presence of CO_2, germ tubes could be formed abundantly in both liquid and solid media, even at 25°C. Germ tubes (although possibly pseudohyphal) were also reported to be formed at 28°C in liquid media, in the absence of exogenous CO_2 in two previous reports (Hrmová & Drobnica, 1981, 1982).

However, a number of questions remained. Sims (1986) only reported whether there was a "grossly and unequivocally greater" mycelial growth in 31 different strains over an 18 h period. There were no data as to the percentage of cells forming germ tubes or how early they were formed. He was also only able to demonstrate germ tube formation at 25°C using horse serum and not with the other amino-containing compounds tested. Finally, he claimed that dissolved CO_2 was required for germ tube formation since bicarbonate could not replace CO_2 and because when C. albicans was grown on plates under a cover slip and in a 10% CO_2 atmosphere, hyphae appeared more abundantly in the uncovered areas.

Our present results demonstrate that there are a number of factors controlling germ tube formation at temperatures below 37°C. In contrast to Sims (1986), we found that gaseous CO_2 could be replaced by bicarbonate. However, while we did not have to alter the atmospheric conditions, we cannot discount the possiblility that dissolved CO_2 produced by solubilization of

bicarbonate is actually responsible for the dimorphic change. The requirement for an adequate depth of the media in the tubes and the fact that they could not be continuously shaken, tend to support the notion that dissolved CO_2 is the active form or is, at the very least, required. Certainly at the lower pH values bicarbonate is most likely to have converted to gaseous CO_2. However, it is also possible that removal of CO_2 gas by shaking upsets the equilibrium and lowers the concentration of bicarbonate below a critical threshold. Furthermore, at the higher pH values it is more likely that bicarbonate is the form available to the cell. While it is generally accepted that only aqueous CO_2 can diffuse through the cell membrane (Gutknecht et al., 1977; Jones & Greenfield, 1982), bicarbonate is thought to be able to be transported by a carrier-mediated facilitated diffusion (Miller & Colman, 1980; Lucas et al., 1983; Volokita et al., 1984; Holthuijzen et al., 1987). Both aqueous CO_2 and bicarbonate are known to have membrane and enzyme effects (Jones & Greenfield, 1982) . There has been a report that all three forms of carbon dioxide (CO_2, HCO_3^-, and $CO_3^=$) mediate pH-regulated dimorphism (Bedell, cited in Soll, 1985b). Clearly, which form(s) of CO_2 are responsible for dimorphism remain to be elucidated.

The next important factor appeared to be the concentration of the inducer. However, here the requirement differed depending on the inducer used. A high concentration of proline, but not GlcNAc, was required for germ tube formation at either 30° or 25°C. The concentration of proline required compares favorably with the approximately 70 mM asparagine or glutamine that Sims (1986) used at 30°C. Hrmová & Drobnica (1982) used 100 mM GlcNAc to obtain germ tube formation at 28°C; however, they did not mention if that amount was actually required. We do not know why such a

large amount of proline is required. One interpretation would be that proline is acting as a substrate for an enzyme with very low activity at temperatures below 30°C. The fact that, at least at 30°C, the addition of 0.05 to 5.0 mM glucose reduced the required concentration of proline to normal levels, also supports the idea that at the lower temperatures proline transport is inhibited. Enhancement of amino acid transport by glucose has been reported (Van Mulders, et al., 1983). We have previously suggested (Pollack & Hashimoto, 1985), as have Shepherd et al. (1985), that the cells have adequate endogenous nitrogen reserves and that they only require exogenous carbon. Shepherd and co-workers have further advanced the idea that the inducer merely interacts with a surface receptor, thereby triggering an intercellular message (Sullivan & Shepherd, 1982; Shepherd & Sullivan, 1983, 1984). However, their hypothesis does not explain why the combination of proline and glucose does not result in germ tube formation at 25°C. Either explanation would, thus, question whether germ tube formation can proceed without the metabolism of the inducer, at least with regard to proline.

The role that priming plays is also unclear. We previously reported that priming cells before induction by ethanol removes the requirement for bicarbonate in the induction solution, but is not an absolute requirement for ethanol-induced germ tube formation (Pollack & Hashimoto, 1985). Priming also facilitates the induction of germ tubes by low concentrations of ethanol (data not shown). Priming, in the present report, also, apparently reduced the amount of bicarbonate required to be added for germ tube formation to occur, and allowed germ tube formation to proceed in about 50% of the cells without addition of bicarbonate at 30°C. Our results appear to be similar to those of Hrmova & Drobnica (1982, 1983) who were able to obtain germ tubes

at 28°C only when the cells were first grown for 96 h at 28°C. It is also well known that under certain growth conditions only stationary phase cells can be induced to form germ tubes (Chaffin & Sogin, 1976; Soll, 1984, 1985a, 1985b). On the other hand, Soll & Herman (1983) claimed that the requirement that the cells be in stationary phase could be superseded by starvation for only a short period. Such short periods did not enhance germ tube induction by GlcNAc at 30°C. It would appear, then, that priming allows germ tube formation to occur under suboptimal conditions. Why priming was required for GlcNAc and not for proline is unknown. It is possible that proline is assimilated more readily than GlcNAc and that priming may facilitate the uptake of inducer/nutrient. Priming may also increase the internal bicarbonate/carbon dioxide reserves.

Hrmová & Drobnica (1981) showed that concentrations of glucose greater than 5 mM inhibit germ tube formation at 28°C; however, when germ tubes were induced by the antibiotic monorden, glucose concentrations as high as 100 mM were without effect (Hrmová & Drobnica, 1982). Our results show that even 1 mM glucose suppressed, but did not completely inhibit, germ tube formation at 25°C, while 400 mM had no effect at 30°C. As discussed in our previous paper (Pollack & Hashimoto, 1987), the reason for suppression by glucose remains to be clarified.

These results demonstrate that germ tube formation in C. albicans is regulated by a delicate balance between a number of factors, especially when it occurs at conditions other than optimal. In addition, the fact that a wild-type strain of C. albicans is capable of forming germ tubes at low temperatures, may help in determining the mechanism by which recently reported mutants, are able to form germ tubes at 25°C under standard conditions (Bedell

& Soil, 1979; Buckley et al., 1982; Hubbard et al, 1986).

CHAPTER VI

TEMPERATURE REGULATION OF GERM TUBE FORMATION IN *CANDIDA ALBICANS*

ABSTRACT

Commitment studies were used to assess temperature regulation of germ tube formation in *C. albicans*. More than 80% of the cells formed germ tubes 5-25 m long by 4 h at 25°C in the presence of a nutrient inducer (proline, GlcNAc, 4 mM; or ethanol, 17.1 mM), following incubation in inducer-free buffered salts at 37°C for 25 min. Exposure to 37°C in the presence of an inducer for up to 1 h, followed by removal of the inducer by filtration and reincubation in buffered salts, resulted in the formation of germ tubes only 5 m long, by 4 h. In all instances, bicarbonate had to been exogenously supplied in order for germ tube formation to occur. There did not seem to be any significant difference in the rate of uptake of either proline or GlcNAc from the medium under germ-tube-forming and non-germ-tube-forming conditions. The results indicate that induction at 37°C serves to remove many of the requirements for germ tube formation at lower temperatures, but does not do so by regulating the rate of uptake of the nutrient inducer. In addition, bicarbonate and the inducer may be "hyphal growth factors" instead of merely initiators of the dimorphic process.

INTRODUCTION

Recent findings have shown that a temperature of 37°C is not absolutely required to form germ tubes in Candida albicans. Rather, the 37°C temperature requirement can be replaced by either an atmosphere of 10% carbon dioxide (Sims, 1986) or by incubation, without shaking, in a medium containing 2 mg/ml bicarbonate and with a high concentration of the nutrient/inducer (Pollack and Hashimoto, in preparation). The question thus, arose as to the exact role a temperature of 37°C plays in germ tube formation, and why it has been the most universally accepted of all previously reported requirements for germ tube formation. In order to better understand the situation, we looked at temperature-regulated commitment to form germ tubes. The results demonstrated that induction at 37°C serves to remove, or at least reduce, many of the requirements for germ tube formation at lower temperatures, but does not do so by regulating the rate of uptake of the nutrient inducer. In addition, bicarbonate and the inducer may be "hyphal growth factors" instead of merely initiators of the dimorphic process.

METHODS

Organism. Candida albicans (ATCC 58716) was used throughout this study. Cells were maintained on Sabouraud dextrose agar (SDA) at 25°C.

Growth and preparation of cells. Cells were grown for 24 to 32 h on SDA at 37°C. They were then washed 10 times by filtration on a Millipore filtration apparatus (Filter size 0.45 µm), being careful to resuspend the cells between each washing by suction and ejection through a pasteur pipette. These cells, used immediately were termed non-primed cells. In some instances it was necessary to "prime" the cells by incubating them in water at 20°C for an additional 16 to 24 h, in order to obtain optimal germ tube formation.

Induction of germ tubes. Washed yeast cells (8×10^5 ml^{-1}) were suspended in a buffered salts solution (initial volume 4 to 10 ml), at either 37°C or 25°C. Proline or N-acetylglucosamine (4 mM final concentration) were added either at the beginning of the incubation to this solution, or at some time after the commencement of the incubation, after 0.375 ml aliquots were removed from the original solution. The composition of the buffered salts solution has been detailed previously (Pollack & Hashimoto, 1987). In some experiments, the inducer was removed by first washing as described above, then replacing the filter and washing an additional 4 times to eliminate any carryover. The cells were incubated for 4 h after the final transfer at 25, 30 or 37 °C, without shaking. The cells were then examined by phase microscopy for percentage germ tube formation.

Measurement of uptake of proline and GlcNAc. Uptake of proline and GlcNAc

was determined by measuring the decrease in the concentration of each compound from an incubation solution containing 1.5 X 10^7 cells ml^{-1}) and 0.8 mM proline or GlcNAc. To determine the effect of 37°C incubation temperature, the cells were incubated in buffered salts (pH 7.0) for 40 min at 37°C or 25°C. Both sets of cells were subsequently incubated at 25°C, at which time 0.8 mM proline or GlcNAc was added. Samples were removed at regular intervals, filtered to remove the cells, and analyzed colorometrically for the concentration of proline remaining, using the method of Blumenkrantz (1980), or for GlcNAc remaining, using the method of Reissig et al. (1955). To determine the effect of bicarbonate, uptake of GlcNAc at 30°C was measured from an incubation solution containing, or lacking, sodium bicarbonate (2 mg ml^{-1}).

RESULTS

Minimum length of exposure to 37°C required for germ tube formation

Maximum germ tube formation cold be obtained after only 20 to 30 minutes of exposure to 4 mM proline at an induction temperature of 37°C. Only 20 min at 37°C was sufficient for 70% of the cells to become committed to form germ tubes by 4 h, while following 30 min exposure to 37°C, approximately 90% of the cells were committed to forming germ tubes (Fig. 21). However, proline did not have to be present during the incubation at 37°C. Essentially the same curve was obtained when the cells were incubated in a buffered salts solution, without proline, at 37°C, then adding proline (4 mM) after the cells were transferred to 25°C, where they were incubated for an additional 4 h (Fig. 21). It should be noted that at this concentration of proline (4 mM) germ tube formation does not occur at 25°C. In addition, proline could be replaced by 4 mM GlcNAc, 0.1% ethanol, or 5 mM glucose, with essentially the same results. None of these compounds are capable of inducing germ tube formation at 25°C. This activated state of the cells was gradually lost by incubation in buffered salts at 25°C, although 50% of the cells were still capable of forming germ tubes after 3 h (Fig. 22). Continued incubation at 37°C in buffered salts past 75 min also resulted in loss of activation (data not shown). The observation that a short incubation at 37°C in buffered salts, followed by the addition of proline at 25°C, occurred only when the volume of the initial solution was greater than 2 ml. If the volume of the buffered salts was 0.5 ml, only 25% of the cells formed germ tubes. However, this value could be significantly increased if additional bicarbonate were added along with the proline (Table 18, see further). Although there

Fig. 21. Influence of time exposed to 37°C on commitment to germ tube formation. Cells were incubated at 37°C in buffered salts, pH (7.0), either containing (-●-) or lacking (-O-), 4 mM proline, for increasing periods of time. At the time indicated on the abscissa cells were transferred to 25°C, and 4 mM proline was added to the cells previously incubated in buffered salts only. The percentage of cells forming germ tubes was determined after 4 h exposure to proline. Values are the means of three experiments.

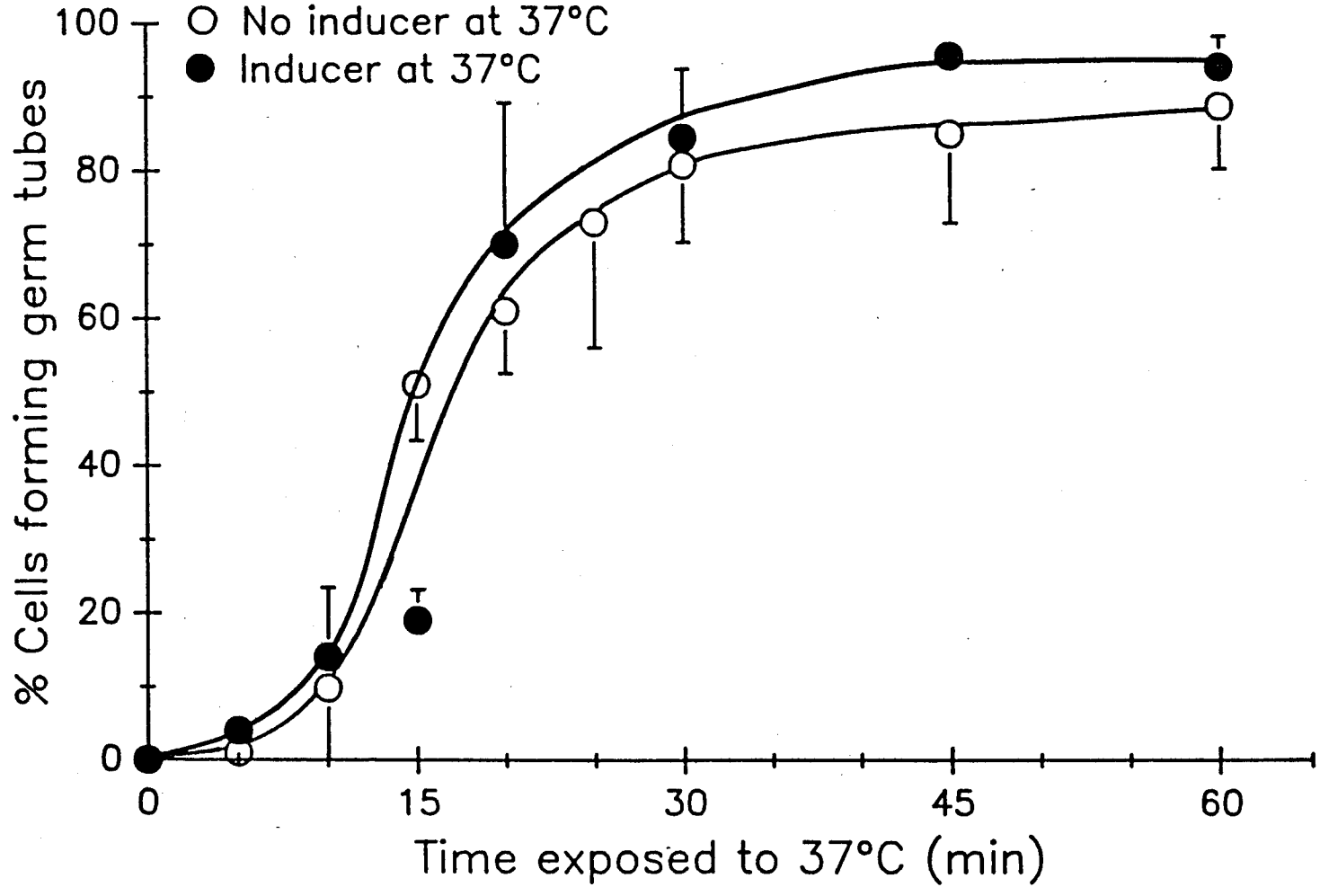
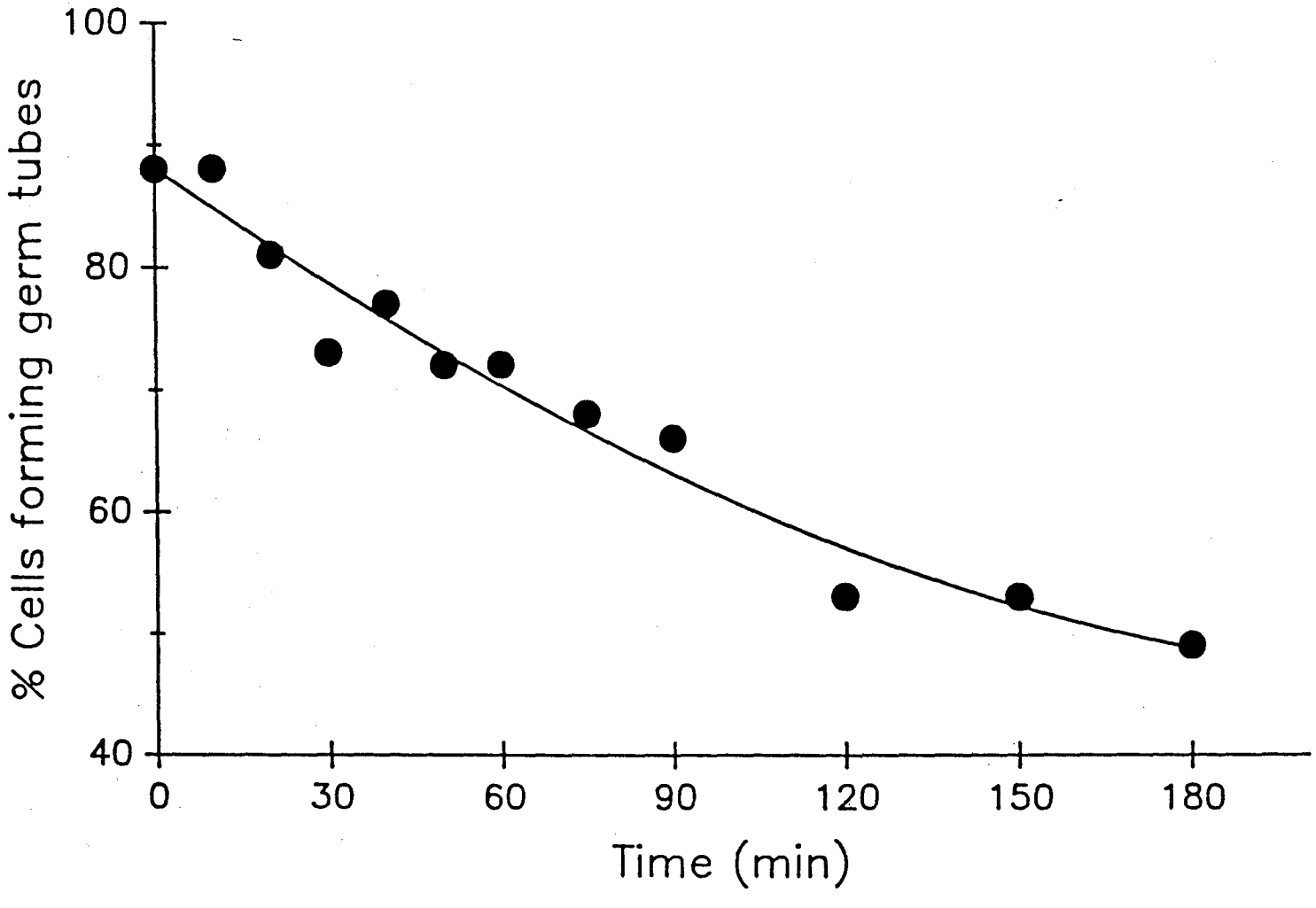

O No inducer at 37°C
● Inducer at 37°C
% Cells forming germ tubes
100
80
60
40
20
0
0
15
30
45
60
Time exposed to 37°C (min)

Fig. 22. Loss of commitment to form germ tubes following exposure to 37°C. Cells were incubated in buffered salts (pH 7.0) at 37°C for 40 min, after which they were transferred to 25°C. Proline (4 mM) was added to the cells at the times, indicated on the abscissa, after the cells were transferred to 25°C. The percentage of cells forming germ tubes was determined after 4 h exposure to proline.


100
80
60
40
% Cells forming germ tubes
0
30
60
90
120
150
180
Time (min)

was significant background germ tube formation when the cells were incubated for only 60 min in buffered salts in a volume of 2 ml of greater (37%), there was still a significant increase in this value when proline was added subsequent to the transfer to 25°C. In addition, the germ tube length also increased significantly from 6 to 18 μm (Table 18).

Effect of removal of inducer from the incubation solution

Having demonstrated that the temperature and inducer requirements need not be simultaneous in order to obtain germ tubes, we wished to ascertain what would result if the inducer was removed after the cells were minimally exposed to it. As shown in Table 18, the major effect of removal of the inducer was on germ tube length. Constant exposure to 4 mM proline, even when the incubation temperature was reduced from 37°C to 25°C after 40 to 60 min, resulted in germ tubes as long as 40 μm by 5 h. However, relatively short germ tubes, 2 to 15 μm long, were formed when proline was removed by filtration after 60 min followed by reincubation in buffered salts only for 4 h. Thus, proline was required as a nutrient for germ tube elongation.

The requirement for bicarbonate for germ tube elongation

The addition of bicarbonate is not necessary for germ tube formation at 37°C, but is an absolute requirement below 30°C (Chapter 5). When the cells were induced by an initial 60 min exposure to 37°C in the buffered salts solution, lacking proline, bicarbonate did not have to be present during the initial period. However, if the bicarbonate was also lacking during the subsequent growth period at 25°C in the presence of proline, very few cells formed

Table 18. The role of preincubation conditions in commitment to form germ tubes.

Sequence of incubation conditions		Percentage of cells forming germ tubes	Germ tube length
1st 60 min	Next 4 h		
37°C, Pro, BSC[1]	25°C, Pro, BSC	94.0 ± 4.2	22.3 ± 9.9
37°C, BSC(>2 ml)[2]	25°C, Pro, BSC	78.5 ± 11.7	18.0 ± 6.2
37°C, BSC(0.5 ml)	25°C, Pro, BSC (+ HCO_3^-)[3]	24.8 ± 8.6 (60.5 ± 8.5)	N.D.
37°C, BSC (>2 ml)	25°C, Glc[4], BSC	70.9 ± 9.6	18.8 ± 6.3
37°C, BSC (>2 ml)	25°C, BSC	36.8 ± 7.7	6.1 ± 6.0
37°C, BS[5] (>2 ml)	25°C, Pro, BSC	63.4 ± 8.2	14.2 ± 6.4
37°C, BS	25°C, Pro, BS	4.3 ± 2.8	N.D.
37°C, Pro, BSC wash 10X[6]	37°C, BSC (0.5 ml)	59.5 ± 6.4	3.9 ± 3.2
37°C, Pro, BSC wash 10X	25°C, BSC	43.2 ± 9.0	3.8 ± 3.0
37°C. Pro, BSC wash 10X	37°C, BS	49.0 ± 9.0	4.8 ± 3.1
37°C, Pro, BSC wash 10X	25°C, Glc, BSC (0.5 ml)	69.7 ± 9.0	13.5 ± 3.6
25°C, Pro, BSC wash 10X	37°C. BSC (0.5 ml)	12.6 ± 9.9	N.D.
37°C, BSC (0.5 ml)	37°C, BSC	6.1 ± 6.1	N.D.

1 Cells were incubated for 60 min at 37°C, in buffered salts, containing 2 mg/ml sodium bicarbonate (BSC), and 4 mM proline (Pro), after which they were transferred to 25°C and incubated without shaking, for 4 additional h. The final volume during

Table 18 (cont.)

the final 4 h incubation in all experiments in this table was 0.5 ml. Values in this table are the means ± SD of at least three experiments.

2 Cells were incubated for 60 min at 37°C, in buffered salts, containing 2 mg/ml sodium bicarbonate (BSC), after which they were transferred to 25°C, when 4 mM proline was added. Similar results were obtained if 4 mM GlcNAc, 0.1% ethanol, or 5 mM each of glucose and NH_4Cl were substituted for proline.

3 An additional volume of 0.05 ml containing 8 mg/ml sodium bicarbonate was added when the proline was added to the buffered salts solution.

4 Glucose (5 mM)

5 Cells were incubated for 60 min in buffered salts only (BS), after which they were transferred to 25°C, when 4 mM proline was added.

6 Cells were incubated for 60 min at 37°C in BSC containing 4 mM proline, after which they were washed 10X by filtration to remove the proline, and then reincubated at 37°C in BSC only.

germ tubes (Table 18). The lack of bicarbonate during the initial 60 min exposure to 37°C also had no effect on the subsequent length of the germ tubes, as long as it was present during the subsequent 4 h incubation period.

The effect of temperature and bicarbonate on uptake of the inducer

Uptake of proline and GlcNAc at 25°C was measured following a 40 min exposure of the cells to 37°C in buffered salts. Proline was immediately taken up by the cells, while there was a 45 to 60 min lag period before GlcNAc was taken up. In both cases, though, there was no difference in the uptake of cells initially exposed to 37°C (germ-tube-forming conditions) and those exposed to 25°C continuously (budding conditions)(Figs. 23 and 24).

Uptake of GlcNAc by cells incubated in the presence of 2 mg ml^{-2} bicarbonate at 30°C is shown in Fig. 25. There was a lag period of about 30 min before uptake occurred, followed by continuous uptake for the duration of the 4 h measurement period. Again there was no difference in the rate of uptake between those cells exposed to bicarbonate (germ-tube-forming conditions) and those not exposed to bicarbonate (budding conditions). Uptake of proline at 30°C or 25°C were not attempted due to the high concentration of proline required for induction of germ tubes at those temperatures. However, under all other conditions tested, (such as in the presence and absence of glucose at pH 3.8; Pollack and Hashimoto, 1987) no distinction was ever detected in the uptake of proline or GlcNAc between germ tube and yeast forming conditions (data not shown).

Induction of germ tubes by analogs of proline and GlcNAc

Since elongation of the germ tubes into hyphae required the continued

Fig. 23. Comparison of proline uptake from the incubation solution following exposure of cells to 37°C with uptake from solution not exposed to 37°C. Cells (1.5 X 10 ml^{-1}) were incubated in buffered salts (pH 7.0) for 40 min at 37°C (-O-) or 25°C (-●-). Both sets of cells were subsequently incubated at 25°C at which time 0.8 mM proline was added. Samples were removed at regular intervals, filtered to remove the cells, and analyzed for the concentration of proline remaining using the method of Blumenkrantz (1980).

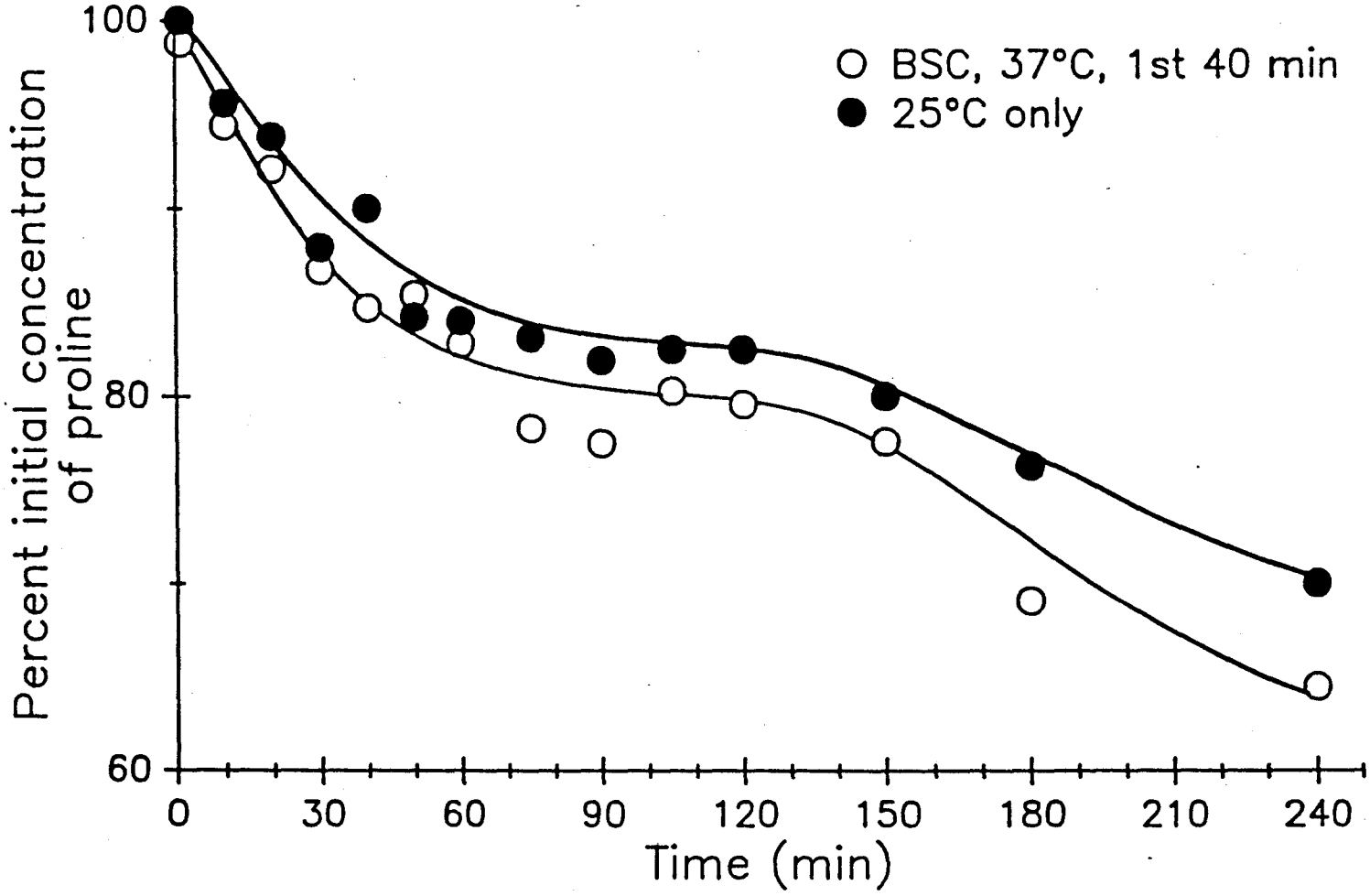

100
80
60
Percent initial concentration of proline
BSC, 37°C, 1st 40 min
25°C only
0
30
60
90
120
150
180
210
240
Time (min)

Fig. 24. Comparison of N-acetylglucosamine uptake from the incubation solution following exposure of cells to 37°C with uptake from solution not exposed to 37°C. Cells (1.5 X 10^7 ml^{-1}) were incubated in buffered salts (pH 7.0) for 40 min at 37°C (○) or 25°C (●). Both sets of cells were subsequently incubated at 25°C at which time 0.8 mM GlcNAc was added. Samples were removed at regular intervals, filtered to remove the cells, and analyzed for the concentration of GlcNAc remaining using the method of Reissig et al. (1955).

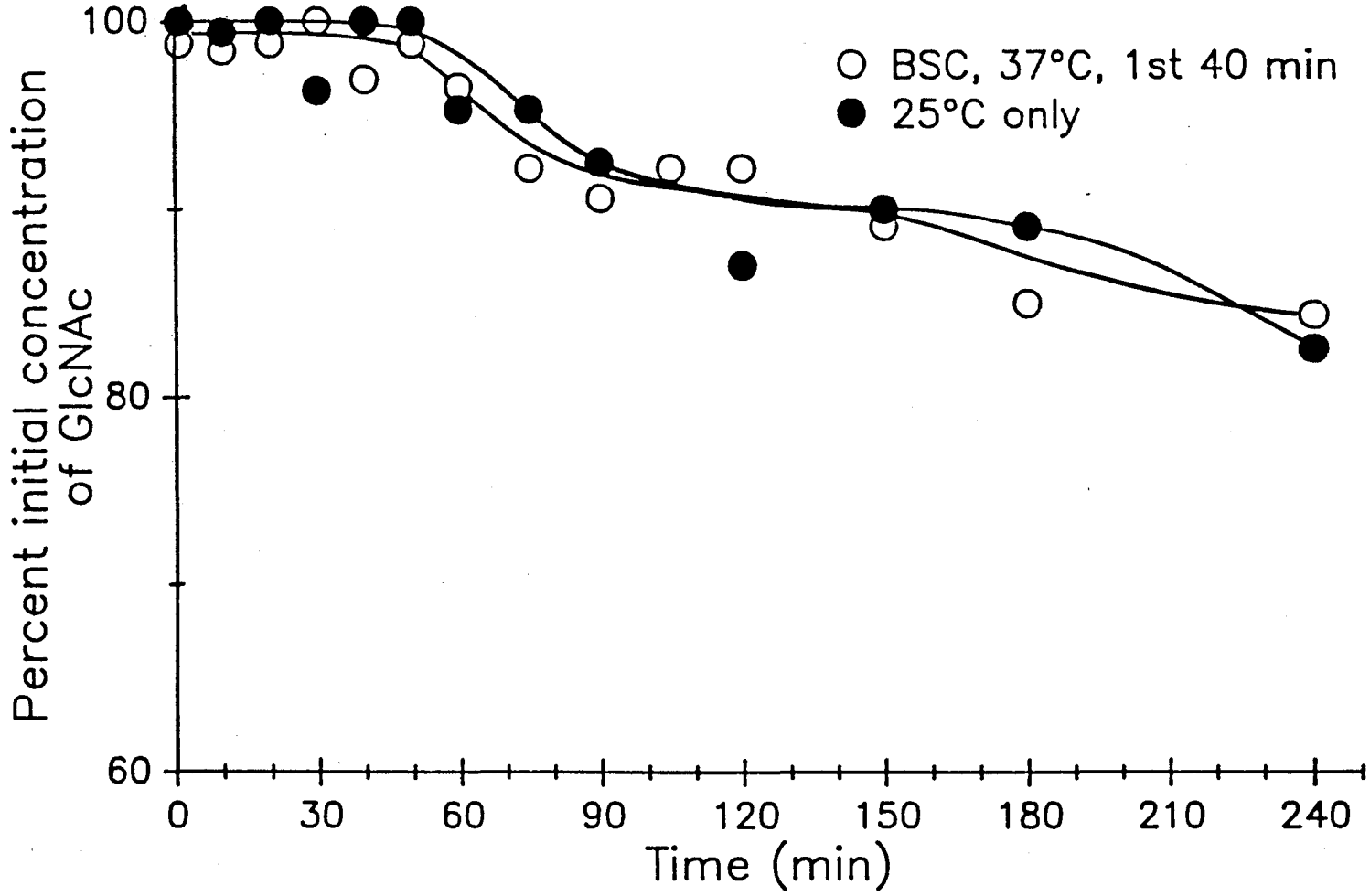
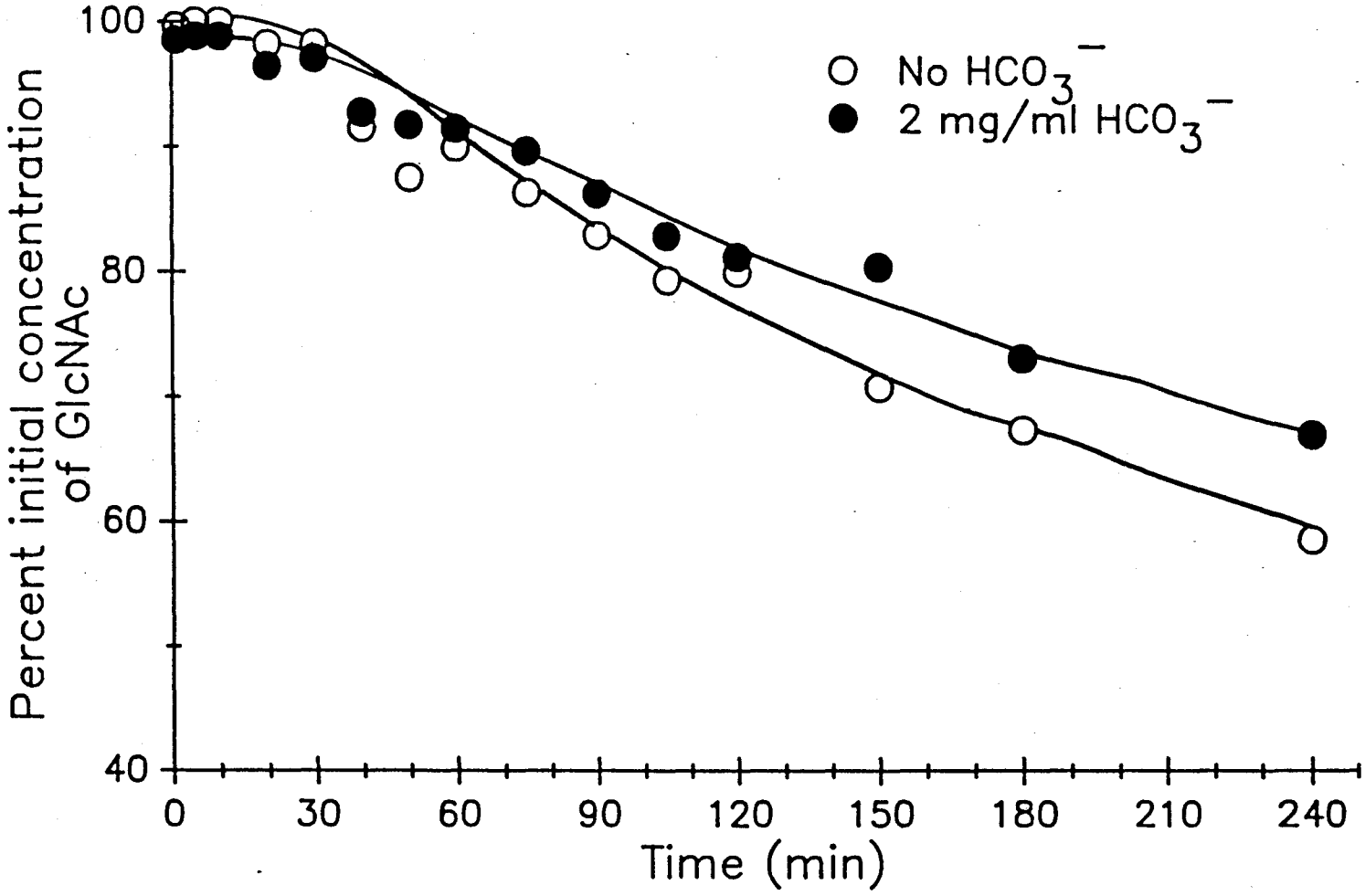

100
80
60
Percent initial concentration of GlcNAc
BSC, 37°C, 1st 40 min
25°C only
0
30
60
90
120
150
180
210
240
Time (min)

Fig. 25. Comparison of N-acetylglucosamine uptake at 30°C from an incubation solution containing bicarbonate with uptake from a solution lacking bicarbonate. Cells (1.5 X 10^7 ml^{-1}) were incubated at 30°C in buffered salts (pH 7.0) containing 0.8 mM N-acetylglucosamine and either containing 2 mg/ml sodium bicarbonate (●) or not containing any bicarbonate (○). Samples were removed at regular intervals, filtered to remove the cells, and analyzed for the concentration of GlcNAc remaining using the method of Reissig et al. (1955).


100
80
60
40
Percent initial concentration of GlcNAc
No HCO_3^-
2 mg/ml HCO_3^-
0
30
60
90
120
150
180
210
240
Time (min)

presence of proline or GlcNAc, and since, in the case of proline, very high concentrations were required for germ tube formation to occur, it is possible that the inducers must be metabolized. This conflicts with the evidence of Shepherd and Sullivan (1982; 1983). We therefore attempted to repeat their experiments using different analogs of GlcNAc as inducers, in particular N-acetylglucosamine linked to agarose. We were unable to repeat their results even when using the same strain and the same induction solutions. Few germ tubes were formed by any of the listed inducers in the presence of glucose, as they reported. Likewise, analogs of proline, except for proline esters, were also incapable of inducing germ tube formation (Table 5, p. 61).

DISCUSSION

We have shown previously that a number of requirements for formation of germ tubes at 25° to 30°C, are either removed, or greatly reduced, by incubation at 37°C. These requirements include: bicarbonate, no shaking during incubation, priming of the cells, high concentration of and type of inducer, and restrictive pH values (Chapter 5). The present results show that while some of these requirements are removed by a short exposure to 37°C sufficient to commit the cells to forming germ tubes, others remain. Thus, while 200 mM of proline is required to induce germ tube formation at constant exposure to 25°C, a 30 min exposure in buffered salts is sufficient to allow at least 4 mM proline to induce germ tube formation at the lower temperature. The short exposure to 37°C is also sufficient to allow GlcNAc, ethanol, and even the non-inducer, glucose, to induce significant germ tube formation at 25°C. None of these compounds can do so when the cells are constantly incubated at the lower temperature. Similarly, while germ tubes can be induced by proline only at a pH of approximately 7.0, when exposed to 37°C for 30 min at pH 5.8, germ tube formation can proceed when the proline is added at 25°C. On the other hand, the absence of bicarbonate at 25°C, following a 60 min exposure to buffered salts at 37°C, does not allow germ tube formation to occur appreciably. Even the presence of bicarbonate in a small volume (0.5 ml) in the initial solution exposed to 37°C, was insufficient to allow significant germ tube formation to occur, without the further addition of bicarbonate when the cells were transferred to 25°C. This is probably due to the loss of bicarbonate from the shallow solution at the higher temperature. These results seem to indicate that bicarbonate, or carbon dioxide,

is actually a constantly required "hyphal growth factor" which is, perhaps, produced by the cell in sufficient quantities at 37°C, but not at lower temperatures, thus requiring its being supplemented in the growth medium. The observation of a high backround of germ tube formation when the cells were incubated in buffered salts only for 60 min, without any exposure to proline, which would indicate that CO_2 alone can induce germ tube formation, will be dealt with in the next chapter.

The role of the inducer itself is less clear. Apparently, it is required for elongation to occur. Addition of proline at 25°C, following exposure to buffered salts at 37°C, causes an increase of germ tube length from 6 to 18 μm. Likewise, its removal from the induction medium, when the cells continued to be incubated at 37°C, resulted in cessation of elongation. However, once commitment had occurred it could be replaced by glucose, but not by the other non-inducing amino acids found in the medium of Lee et al. (1975). The other amino acids are perhaps poor carbon sources. In fact, once the cells were exposed to buffered salts at 37°C, elongation and increased germ tube formation occurred with the addition of glucose, a compound which no one has ever demonstrated has an inductive capacity. The evidence from Table 18 indicates that proline, and probably the other reported inducers, are good carbon sources, and possibly good generators of CO_2, but are not specific inducers of germ tube formation.

It is not clear which signal is required for the actual induction to occur. Shepherd and Sullivan (1983, 1984; Sullivan and Shepherd, 1982) have suggested, based on results of nonmetabolized or "gratuitous" inducers, that the inducer reacts externally with the cell membrane, and generates a morphogenetic signal. In their system, also, glucose must be supplied to actually

visualize germ tubes. Their hypothesis conflicts with that of Dabrowa and Howard (1981), who suggested that proline metabolism is required based on the observation that a minimum exposure to the inducer of 30 min is required for germ tube formation to commence. This time course is much greater than the 30 s required for bacterial spore germination, which does not require that the inducer be metabolized. Furthermore, analogs of L-proline, such as D-proline, which competitively inhibit uptake of L-proline, also reduce germ tube formation, (Dabrowa and Howard, 1983). Taschdjian and Kozinn (1961) also showed that germ tube formation was inhibited when uptake of free amino acids were inhibited by gamma globulin, although Barlow et al. (1974) questioned whether the free amino acids play a role in germ tube formation. On the other hand, others have shown that substances which stimulate germ tube formation, like albumin, are not measurably consumed (Taschdjian and Kozinn, 1961; Bernander and Ebedo, 1969, Barlow et al., 1974). However, albumin was not the only inducing substance in their media.

We have questioned, whether it is possible that the inducer does not have to be metabolized, based on the observation that at 30°C and 25°C a high concentration of proline (200 to 350 mM) is required for germ tube formation to occur (Chapter 5). It would seem highly unlikely that increasing the concentration of the inducer would be required if all that was required was an externally generated signal. However, high concentrations of GlcNAc were not required at 30°C, so it was possible that Shepherd and Sullivan's hypothesis was unique to the GlcNAc-induced system.

The uptake experiments demonstrated that GlcNAc is an induced system, as significant uptake did not begin for at least 30 min while proline uptake was almost immediate. Since germ tubes begin to emerge in both

proline-induced and GlcNAc-induced systems at about 30 min, little, if any, metabolism of GlcNAc occurs before germ tubes emerge. These results will have to be confirmed using more sensitive radioisotopes to measure uptake and metabolism. It is probably significant that "commitment" to form germ tubes at non-inducing conditions (25°C) did not occur until significant amounts of GlcNAc were assimilated by the cells. However, since the addition of glucose alone at 25°C was sufficient to obtain long germ tubes following the exposure to 37°C, it would seem that GlcNAc is not the specific inducer that Shepherd and Sullivan (1982) claimed it to be. Rather, "commitment," may only be the outgrowth and elongation of germ tubes in the presence of a good carbon source, but which were triggered initially by another factor, possibly bicarbonate.

Our attempts at repeating the results of Shepherd and Sullivan were not successful. These attempts included using their strain of C. albicans and their imidazole buffer and salts solution. The mannosamine used in our experiments was reference grade, while the agarose linked GlcNAc was washed numerous times to avoid any possible contaminants. Recent results from their laboratory have shown that mutant strains with reduced capability to metabolize GlcNAc cannot form germ tubes when induced with GlcNAc (Corner et al., 1986). We therefore believe that careful experiments are still necessary before it is possible to positively conclude whether the metabolism of the inducer is required for the morphogenetic signal to be generated or whether an interaction with the cell surface is sufficient.

CHAPTER SEVEN

CARBON DIOXIDE-INDUCED GERM TUBE FORMATION IN CANDIDA ALBICANS

Carbon dioxide is a recognized mediator of morphogenesis in many bacteria and fungi (Wimpenny, 1969; Jones and Greenfield, 1982). These effects include: stimulating germination of bacterial spores (Holland et al., 1970; Grund and Ensign, 1978) and fungal spores (Yanagita, 1963; Pass and Griffin, 1972), triggering formation of sporangial plants (Cantino, 1956), inhibition of fruit body formation (Sietsma et al., 1977), affecting cell shape and division (Straskrabova et al.,1980; Lumsden et al., 1987), and initiating dimorphism in fungi (Bullen, 1949; Drouhet and Mariat, 1952; Converse and Besemer, 1959; Bartnicki-Garcia and Nickerson, 1962; Houston et al., 1969; Ho and Smith, 1986). It has not, though, been considered a major modulator of dimorphism in Candida albicans.

Recently, Sims (1986) suggested that an increased concentration of CO_2 produced by the metabolism of the organism or by growth in a 10% CO_2 atmosphere mediated germ tube formation in C. albicans. The effect was thought to be physical, perhaps caused by intracellular accumulation of CO_2, since albumin was capable of inducing germ tube formation at 37°C, in the absence of CO_2, without being perceptibly metabolized. It was conjectured that albumin loosely bound atmospheric CO_2 and maintained an environment of increased CO_2 tension about the cells. However, Sims (1986) could only obtain germ tubes when certain nitrogen-containing compounds, generally diamides, were present in the media. Nor, was the addition of 10% CO_2, in the

presence of all albumins, always effective in producing germ tubes. He, therefore, proposed that some reaction between the albumin and CO_2 is required for induction to occur. All of his media also contained 0.1% glucose.

Our earlier research demonstrated a requirement for bicarbonate when germ tube formation was either induced by ethanol at 37°C (Pollack and Hashimoto, 1985) or when induced by proline or N-acetylglucosamine (GlcNAc) at temperatures between 22°C and 30°C (Pollack and Hashimoto, in preparation). In the latter instance, germ tubes were formed only when there was a ratio of surface area to tube depth (SATD ratio) of less than 5:1 mm. This requirement for a minimum depth was thought to be possibly related to retention of CO_2 by the medium. Although there never was a requirement for exogenous bicarbonate when cells were induced with proline or GlcNAc at 37°C, there was the possibility that the metabolism of both inducers supplied the required CO_2 to the cells in sufficient amounts. We, therefore, returned to investigate the effects of CO_2 on germ tube formation at 37°C.

In all of our previous studies at 37°C, we induced germ tube formation in a 0.5 ml solution in a 12 x 75 mm test tube. This resulted in a SATD ratio of 9.8 mm. Although the buffered salts solution contained 0.75 mg ml^{-1} sodium bicarbonate, germ tubes almost never formed unless a nutrient inducer (proline, GlcNAc, ethanol, Dulbecco's modified Eagle's medium, or the combination of NH_4Cl and glucose) was also added to the buffered salts (Pollack and Hashimoto, 1985). Increasing the concentration of bicarbonate to 2 mg ml^{-1}, in the absence of a nutrient inducer, only slightly increased the percentage of cells forming germ tubes. On increasing the volume to 2 ml, though, (SATD ratio of 3.0), and by incubating the cells with minimal agitation, up to 60% (mean 42%) of the cells formed germ tubes 2 to 12 m long (Table 19).

Table 19. Formation of germ tubes in the absence of a nutrient inducer using a test tube system.

Incubation conditions[1]	Volume of tube (ml)	Percentage of cells forming germ tubes
Buffered salts + HCO_3^-		
Strain 58716	0.5	15
	2.0	42
Strain 14053	2.0	80
Strain 10261	2.0	41
Buffered salts		
Strain 58716	2.0	6
Buffered salts + HCO_3^- + glucose		
Strain 58716	0.5	18
	2.0	74

1. Cells (8×10^5 ml^{-1}) were suspended in a buffered salts containing either sodium bicarbonate (1 mg ml^{-1}) or glucose (5 mM), or both, in a 12 x 75 mm test tube and incubated for 4 h at 37°C. The buffers (0.05 M) used included: sodium phosphate (pH 5.8), Tris-(tris[hydroxymethyl]amino methane)-maleate (pH 5.8 and 6.8), HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH 7.0), and MOPS (3-[N-morpholino]propanesulfonic acid) (pH 6.8). The salts used were described in Pollack and Hashimoto (1985). The results were not affected by the different buffers used, nor by the different pH values. Percentage of cells forming germ tubes was determined by counting 100 to 200 cells using a Nikon Diaphot-TMD inverted microscope with a phase contrast lens (40 x).

In order to determine if the volume effect was due to an increase in the concentration of CO_2, the experiment was repeated in a 16 mm diameter tissue culture cluster well using a volume of only 0.15 ml, and incubating the cells under an atmosphere of 5-10% CO_2 in air (model 2210 CO_2 incubator, Queue Systems, Parkersburg, West Virginia). Under these conditions, between 60 and 90% of the cells formed germ tubes using ATCC strains 58716 and 14053. Only 23% of the cells from ATCC strain 10261 formed germ tubes, however (Table 20). Gaseous CO_2, however, although required, was not sufficient by itself for germ tube formation to occur in the tissue culture well system, as bicarbonate also had to be present in the buffered salts solution. In the absence of the 2 mg ml^{-1} bicarbonate germ tube formation was reduced by one-half (Table 20).

The percentage of cells forming germ tubes could be dramatically increased in either the tube or well systems by adding 5 mM glucose to the buffered salts solution. In the presence of 0.75 mg ml^{-1} bicarbonate or less almost no cells form germ tubes when glucose was added to the buffered salts in a volume of 0.5 ml. However, in presence of 1 to 2 mg ml^{-1} bicarbonate in a volume of 2.0 ml, or in the tissue culture system in the presence of CO_2, the addition of glucose resulted in greater than 70% of the cells forming germ tubes in the former system and essentially all cells forming germ tubes in the latter system. Even 99% of ATCC strain 10261 cells formed germ tubes when glucose was present in the buffered salts solution (Tables 19 and 20).

Having demonstrated that gaseous CO_2 could replace the necessity of a high SATD ratio in order to obtain germ tubes in the absence of any organic carbon or nitrogen source, we wished to further determine if CO_2 was also required even in the presence of nutrient inducers. To do this, cells were

Table 20. Formation of germ tubes in the absence of a nutrient inducer in a CO_2 atmosphere.

Incubation conditions[1]	Percentage cells forming germ tubes
Buffered salts + HCO_3^-	
Strain 58716	59
Strain 14053	87
Strain 10261	23
Buffered salts	
Strain 58716	31
Buffered salts + glc + HCO_3^-	
Strain 58716	98
Strain 14053	100
Strain 10261	99
Controls - incubation in air Strain 58716	
Buffered salts + HCO_3^-	21
Buffered salts + glc + HCO_3^-	0

1. A 0.2 ml suspension of cells (8×10^5 ml^{-1}) in buffered salts to which was added either sodium bicarbonate (2 mg ml^{-1}) or glucose (5 mM), or both, was placed in a tissue culture cluster well (16 mm dia.) and incubated for 4 h at 37°C in a CO_2 incubator adjusted to 5 % CO_2 in air. Further details are described in the legend to Table 19.

induced with either 5 mM proline or GlcNAc in buffered salts, in tissue culture wells (final volume 0.5 ml) placed in a desiccator jar containing sodium hydroxide pellets. Only 35% of the proline-induced cells, and 56% of the GlcNAc-induced cells formed germ tubes under those conditions. In another experiment, air was slowly bubbled through a solution of either proline or GlcNAc in buffered salts used to induce C. albicans cells to form germ tubes. Regardless of whether the air was passed over a CO_2 trap of sodium hydroxide pellets, or not, germ tube formation was completely inhibited.

The data presented here are the first unequivocal demonstration that CO_2 is capable of inducing germ tube formation in the absence of any other CO_2-reacting compound, such as albumin, or any other nutrient. In this regard, our results differ from those of Sims (1986), who required the presence of a nitrogen-containing compound in his media in order to obtain germ tube formation. It is thus likely that CO_2, normally produced by the metabolism of what have been referred in the past as "inducers", is the actual inducer of germ tube formation in C. albicans. Preliminary data indicate that proline, GlcNAc and other previously referred to "inducers" are good energy sources for C. albicans, while non-inducing amino acids or amino sugars are poor energy sources. The results also demonstrate that all organic nutrients re-quired for C. albicans to form germ tubes are endogenously stored and that germ tube formation is essentially an endotrophic process, an idea first suggested by Joshi et al (1973, 1975). However, their procedure involved inoculating water, or agar, directly, without prior washing of the cells, so that there is a possibility of medium carry over in their system.

The present demonstration of CO_2-induced germ tube formation is extremely important in understanding the mechanism of dimorphism in C. albicans, since it should now be possible to determine the metabolic fate of the actual trigger, with minimal interference from other compounds. The present results will make it possible to determine if metabolism of the inducer is required for germ tube formation to occur. It is possible that the previously reported requirement for glucose in "gratuitous" germ tube formation induced by N-acetylmannosamine or GlcNAc-agarose (Sullivan and Shepherd, 1982; Shepherd and Sullivan, 1983) may be due to the presence of CO_2 in those systems, either generated by metabolism or by retention of the CO_2 in the viscous solution. Carbon dioxide fixation may be a required step for dimorphic change to occur. CO_2 fixation has been shown to occur in a number of other cases where morphologic change occurs (Yanagita, 1963; Grund and Ensign, 1978). Alternatively, CO_2 may play a role in inducing proteinases or glycosidases required to provide utilizable nutrients for growth.

CHAPTER VIII

CONCLUDING DISCUSSION

Candida albicans has been considered unique among the pathogenic dimorphic fungi, since unlike Blastomyces, Coccidioides, Histoplasma, Paracoccidioides, and Sporothrix, the other most studied, pathogenic genera, it forms hyphae in vivo (or 37°C) and grows as a yeast saprophytically. Since most general theories attempting to explain dimorphism are based on induction of cell wall changes, it is understandable that C. albicans has been considered apart from the others. Nevertheless, the same factors which contribute to, or induce, dimorphic change in the other organisms also contribute to the transition in C. albicans. It is thus surprising that carbon dioxide, which has been studied as an important factor in the dimorphism of other fungi, has almost all but been ignored in C. albicans. However, CO_2 does more than contribute to germ tube formation in C. albicans. The most important finding in this study, was that it was possible to conclusively prove that CO_2 is the sole exogenous requirement for dimorphic change to occur. This was possible due to the endotrophic nature of germ tube formation in C. albicans. It may be considered the actual inducer, or trigger, of germ tube formation. This finding should redirect the approach to the study of dimorphism, not only in C. albicans, but in many other dimorphic fungi.

This study also demonstrated some new factors which affect germ tube formation, and clarified many contradictory findings by showing the interrelationships between the many environmental factors which affect germ tube formation. In particular we have shown that:

1. Ethanol induced germ tube formation.
2. Germ tube formation occurred at pH values between 3.0 to 9.0.
3. Glucose suppressed germ tube formation when the pH was below 5.5, but stimulated elongation when the pH was above that value. A corollary of this finding is that the previous reports of pH-regulation of germ tube formation are misleading, and that at best, pH regulates germ tube formation only in conjunction with glucose.
4. Temperatures in the immediate vicinity of 37°C were not required for germ tube formation. The temperature could vary anywhere from 22 to 43°C, depending on the following factors:
    a. Concentration of inducer. Induction at 22 to 30°C required 350 mM proline to obtain optimal percentage of cells forming germ tubes. The addition of glucose allowed a normal concentration at 30°C but not at 25°C.
    b. Priming of cells. Induction at 30°C with GlcNAc required priming of the cells to obtain optimal percentage of germ tube formation.
    c. Type of inducer. Cells could be induced with proline at 22°C, but no lower than 28°C with ethanol or GlcNAc.
    d. pH. At 30°C with GlcNAc, and 25°C with proline, the pH had to be in the immediate vicinity of 7.0.
    e. Glucose. Glucose suppressed germ tube formation at 25°C, but not at 30°C.
    f. Bicarbonate. Exogenous bicarbonate was required for germ tube formation to occur below 37°C.
5. When bicarbonate was present during the final 4 h incubation at 25°C,

along with an inducer, such as proline, only a short (30 min) pre-exposure to 37°C in buffered salts, was required for germ tube formation. Thus, the temperature and inducer requirements need not be simultaneous. Bicarbonate is also a hyphal growth factor.

6. Removal of the inducers, proline or N-acetylglucosamine (GlcNAc), following induction of germ tube formation, resulted in cessation of elongation.
7. There was no difference in the rate of uptake of either proline or GlcNAc from the induction medium under germ-tube-forming and non-germ-tube-forming conditions.

It was concluded that the nutrient inducers of germ tube formation (proline, GlcNAc, ethanol, and the combination of glucose and NH_4Cl), are likely only serving as excellent carbon sources for the cells. In this capacity, they provide nutrition for elongation at all temperatures, and are probably capable of providing all the CO_2 required for germ tube formation at 37°C.

It is most probable that the aforementioned nutrient inducers must be metabolized in order for germ tube formation to proceed. Since GlcNAc was not appreciably taken up by the cells during the first 30 min of exposure, by which time germ tube formation had begun, by what mechanism the actual inducer (supposedly CO_2) is generated, remains to be proven. The data presented here demonstrated that the cell has sufficient endotrophic reserves to form germ tubes provided that bicarbonate alone is exogenously added. It remains to be determined whether GlcNAc induces CO_2 generation by inducing the cell to metabolize these reserves, or if it is itself sufficiently metabolized to generate the required amount of CO_2. The hypothesis that the inducers

need not be metabolized in order for germ tube formation to occur, certainly should be reevaluated.

A number of general questions remain to be addressed. One is why only a very limited a number of compounds are capable of inducing germ tube formation. We have shown that those amino acids which have been found to induce germ tube formation are those which seem to be readily metabolized by the cell, and which can serve as good growth sources. A survey of the literature will demonstrate that the most potent amino acid inducer, proline, is responsible for a number of morphogenic changes in a variety of organisms.

Proline is the only amino acid capable of stimulating germination of Rhizopus arrhizus and R. stolonifer spores in the absence of phosphate (Weber, 1962; Weber and Ogawa, 1965). If phosphate was present, then ornithine, arginine, asparagine and some hexoses could also stimulate germination, but not as effectively as proline. Compounds structurally related to proline were not able to germinate the spores either. Since these compounds also supported growth, the authors concluded that the non-inducing compounds were not penetrating the spore or that the spore lacked the enzymes necessary to convert glutamic acid to proline.

Proline has also been shown to induce germination in a number of species of Aspergillus. Yanagita (1957, 1963) showed that proline, which could induce germination in A. niger, is metabolized to CO_2, which in turn is fixed into the nucleic acid fraction of the cells. Miller (1962) showed that proline allowed the germination of A. niger at 21°C, instead of the normal 30°C, which indicated that the cells could themselves supply a nutrient at 30°C, but not at 21°C. Pass and Griffin (1972) reported that proline, alanine, or glucose plus NH_4Cl were the most effective in supporting germination of A.

flavus conidia. They also found that metabolism of CO_2 was associated with germination since the passing of air through liquid cultures to remove CO_2, inhibited germination.

Griffin et al. (1986) found that Hypoxylon mammatum, the agent of stem canker in aspens, grew fastest with proline as its nitrogen source. They thought that proline was a favored N source of phytopathogens but generally a poor source for saprobic fungi. They further hypothesized that this might be related to the known fact ammonium, glutamate, and glutamine are generally the preferred nitrogen sources for the growth of fungi, and that these compounds repress the utilization of other nitrogen sources (Marzluf, 1981).

Mucor rouxii and M. racemosus are perhaps the model organisms for CO_2-controlled dimorphism. Until recently, it was thought that yeast formation occurred only under anaerobic conditions, in media containing at least 30% CO_2 (Bartnicki-Garcia and Nickerson, 1962a, 1962b; Bartnicki-Garcia, 1963; Phillips and Borgia, 1985). In the presence of glucose (greater than 0.28 M) the requirement for CO_2 under anaerobic conditions for induction of yeast growth, is removed. This was not thought to be due to the generation of metabolic CO_2, but rather to an inhibitory metabolite, since there was no difference in the generation of CO_2 by either high or low hexose concentrations (Bartnicki-Garcia, 1968). Bartnicki-Garcia did not discount the possibility, though, that the presence of glucose reduced the requirement for exogenous CO_2, so that the metabolically produced CO_2 was sufficient to induce germ tube formation. Leija et al. (1986) have now shown that aerobic conditions can also support yeast morphogenesis in Mucor, if one of the following amino acids were available as the nitrogen source: proline, glycine, glutamate, valine or aspartate. Other amino acids, including glutamine, sup-

ported hyphal growth. Growing in the yeast form on aspartate, M. rouxii consumed less O_2 and glucose, released less CO_2, and produced more ethanol, than when grown on leucine and producing hyphae. However, in the final analysis, they thought that the final form was dependent on pH. A decrease in pH correlated with the appearance of hyphae, while keeping the pH constant resulted in the morphology being dependent on the amino acid in the growth medium.

In all of the above cases, the limited inducers, including proline, were metabolized, with the production of CO_2. Although there apparently is no agreement that CO_2 is the ultimate determinant of morphogenesis in these cases, it is certainly implicated.

In C. albicans, Land et al. (1975b) found that CO_2 generation in cells induced by proline was four times less at 30 min, and 10 times less at 2 h, than production by yeast cells grown on NH_4Cl. Ethanol production was immediate in the hyphae, but was not produced in yeast cells until 2 h. Although we never measured CO_2 production, we did find that ethanol production was dependent on the presence of glucose, which was also present in the medium of Land et al. (1975b). No ethanol was produced when cells were incubated in proline at pH 3.8 or in GlcNAc at pH 5.8. Cells (2.5×10^7 cells/ml) induced with GlcNAc at pH 3.8 produced small amounts of ethanol (0.1 mM) within 2 h, as did cells grown in proline and 200 mM glucose. Cells incubated with GlcNAc and 200 mM glucose at either pH 3.8 or 5.8, produced 10- to 14-times more ethanol (data not shown). Though, we also demonstrated that the removal of CO_2 from the incubation solution containing cells being induced by proline inhibited germ tube formation, the exact role of CO_2 is not very clear. For example, more CO_2 could be produced in the presence

of glucose at low pH (as more ethanol is produced) than in the absence of glucose. Yet, germ tube formation is suppressed at the former condition.

Torosantucci et al. (1984) have looked at the amino acid pools in yeast and germ tube-producing C. albicans cells. They found that glutamine and glutamate account for 62% of the total free amino acid in the hyphal cells as opposed to 41% in the yeast cell. Proline is only a minor component of both cell types. This result would seem to indicate the importance of the metabolism of proline to glutamate in germ tube formation. Unfortunately, this result was only found in GlcNAc-induced cells. They reported that there were qualitative differences when the cells were induced with Lee's medium, but did not elaborate, other than to say that the pool size was twice as large. Another inducer of germ tube formation is the combination of glucose and NH_4^+. Ammonium, in the presence of glucose, has been reported to reverse the Pasteur effect (inhibition of utilization of carbohydrates in the presence of O_2) and greatly increase CO_2 production via glycolysis (Lloyd et al, 1983). This was thought to be due to the activation of phosphofructokinase. A related observation was the stimulation by NH_4^+ of ethanol production (Devine and Slaughter, 1980; Harding et al., 1984). Our observation that glucose could support germ tube formation only with the addition of bicarbonate may be related to this phenomenon. Perhaps insufficient glucose is produced through the normal catabolic pathways. The addition of CO_2 exogenously, or the addition of NH_4Cl which would result in more CO_2 production, would then induce germ tube formation.

It would thus appear that if CO_2 production is what regulates germ tube formation, then its production may come from either glycolysis (GlcNAc and the combination of NH_4^+ and glucose) or via the citric acid cycle (proline

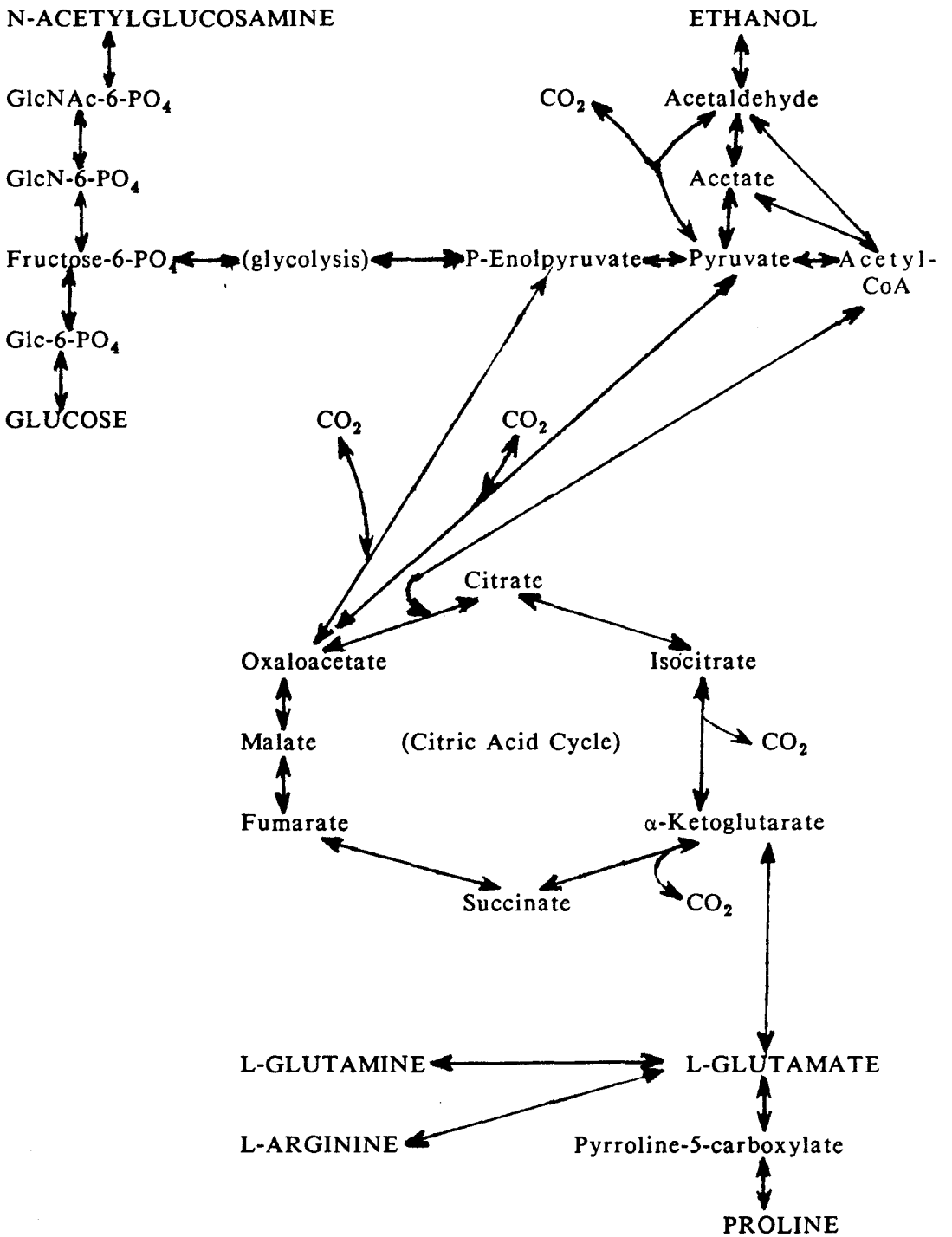
or ethanol). The various pathways for the catabolism of the different inducers are shown in Fig. 26.

A different approach was recently published by Hayashi et al. (1987). They showed that proline was metabolized, in part, to GlcNAc in C. albicans. When they followed labelled proline into the various cell fractions, they found that 1.8% of the label appeared in the chitin fraction. Thus, they argued that GlcNAc is the actual inducer and that proline metabolism into chitin was the reason that proline could also induce germ tube formation. However, the amount of incorporation into chitin appeared too be to small to justify such a claim.

Amino acid control of morphogenesis is not limited to fungi. A limited number of amino acids also control sphere-rod morphogenesis in the bacterium Arthrobacter crystallopoites (Ensign and Wolfe, 1964). Interestingly, this sphere to rod morphogenesis could be induced by biotin deficiency (Chan, 1964; Wills and Chan, 1978), a factor also found to induce hyphal formation in C. albicans (Yamaguchi, 1974a). Since this morphogenic change is assumed to be due to changes in the wall composition (Krulwich et al., 1967) it may be assumed that the metabolism of the inducer is involved.

Such is not the case with bacterial spore germination. A number of studies have shown that proline or other inducers are not metabolized before triggering the germination process (Scott and Ellar, 1978a, 1978b; Rossignol and Vary, 1979a) by interacting with the spore coat Rossignol and Vary, 1979b; Kutima and Foegeding, 1987). Likewise, in neural tissue, amino acid transmitters act by opening ion channels and not through their being metabolized (Usherwood, 1978; Iverson, 1984). Sullivan and Shepherd (1982; Shepherd and Sullivan, 1983, 1984) have argued similarly that the inducer is "gra-

Fig. 26. Pathways for the production of CO_2 by the different nutrient inducers. Capitalized compounds are known inducers of dimorphism in Candida albicans.


N-ACETYLGLUCOSAMINE
GlcNAc-6-PO4
GlcN-6-PO4
Fructose-6-PO4
(glycolysis)
P-Enolpyruvate
Pyruvate
Acetyl-CoA
Glc-6-PO4
GLUCOSE
ETHANOL
CO2
Acetaldehyde
Acetate
CO2
CO2
Citrate
Oxaloacetate
Isocitrate
Malate
(Citric Acid Cycle)
CO2
Fumarate
α-Ketoglutarate
Succinate
CO2
L-GLUTAMINE
L-GLUTAMATE
L-ARGININE
Pyrroline-5-carboxylate
PROLINE

tuitous" and need not be metabolized. We have previously discussed why our evidence questions their conclusion with regard to the standard inducing compounds. However, is it possible that CO_2 itself acts as a trigger, interacting with some receptor, to induce dimorphic change? Or, is it possible that some common chemical structure (for example the carbonyl group), shared by the different inducers, triggers the metabolism of the endogenous reserves? A look at the literature will show that this is possible, but not probable.

Yanagita (1963) demonstrated that during CO_2-induced germination of A. niger spores, CO_2 is fixed into the polysaccharide and protein fraction, followed by the nucleic acid fraction. Later work with A. oryzae showed that the CO_2 was initially incorporated into malate, or possibly oxaloacetate, and that the subsequent labelling of glutamine implied that its synthesis may be one of the key reactions in the germination process (Tsay et al., 1967). Grund and Ensign (1978) showed that CO_2 reacts with pyruvate or phosphoenolpyruvate to form oxaloacetate. Although CO_2 is also taken up by germinating spores and incorporated into protein and RNA, since TCA cycle inhibitors suppress while folic acid antagonists do not, the incorporation into oxaloacetate was considered to be critical to germ tube formation. This was also supported by the observation that oxaloacetate was the only compound able to replace the CO_2 requirement for spore germination. Their conclusion was that CO_2 is involved in the anaplerotic stimulation of the TCA cycle. Oxaloacetate was also shown to be the primary fixation product in Mucor rouxii during dimorphic transition. The subsequent incorporation into aspartate and glutamate supported the conclusion that CO_2 serves an anaplerotic function (Caste and Hartman, 1977). On the other hand, King and Gould

(1971) found no evidence for CO_2 incorporation in germination of Clostridium sporogenes endospores, which also require CO_2 for germination. Enfors and Molin (1978b) argued that their washing procedure may have removed the incorporated CO_2. They do show that CO_2 inhibition of germination involves the accumulation of CO_2 in the membrane lipid layer, and speculated that this affected membrane fluidity. They further suggested that a similar mechanism may be involved in CO_2 stimulation of germination (Enfors and Molin, 1978a). Dixon et al. 1987) have recently shown that CO_2 is required for synthesis of branched-chain amino acid synthesis in C. sporogenes, and that amino acid precursors could override the requirement for CO_2 (Dixon et al., 1987). The results of Land et al. (1975a, 1975b), following the metabolism of proline, are very similar to those reports just cited for the incorporation of CO_2.

On the other hand, carbon dioxide is known to affect many metabolic and growth functions (Jones and Greenfield, 1982). The mechanism of most of these actions is unknown. It is not improbable that many of these effects are physical, such as the previously cited effect on membrane fluidity. Carbon dioxide is also known to lower the internal pH of many types of cells (Aicken and Thomas, 1975; Thomas, 1976; Nikinmaa et al., 1986). The resulting pH gradient may be a induction signal for morphogenesis. Alternatively, carbon dioxide may interact with an enzyme and thereby stimulate, or inhibit, its activity. In fact CO_2 is known to form carbamates with the free amino groups of proteins, which may be one mechanism for such an interaction. Lacking any evidence for such interactions in C. albicans, though, what actual interactions may occur would be just speculation.

The possibility of a role for the enzyme carbonic anhydrase in this process should be considered. This enzyme catalyzes the reaction of CO_2 +

H_2O <---> HCO_3^- + H^+. It requires bound zinc for its activity and is inhibited by imidazole (Kannan, 1980). Alcohol dehydrogenase also requires zinc and is inhibited by imidazole. With regard to C. albicans, it is well known that the concentration of zinc in the growth medium affects the ability of C. albicans to form germ tubes (Widra, 1964; Yamaguchi, 1975; Bedell and Soll, 1979; Soll et al., 1981; Anderson and Soll, 1984; Soll, 1985b). Likewise, imidazole, as we have shown, is inhibitory to germ tube formation. However, the further study of this enzyme's role will need more precise means for its regulation. First, tris buffers may affect the hydration of CO_2. Tris (tris[hydroxymethyl]aminomethane) titrates carbonic acid so that the reaction is: Tris + CO_2 + H_2O <---> $Tris^+$ + HCO_3^+ (Harrington and Kassirer, 1982). Other buffers may also interfere. We have found that EDTA did not inhibit germ tube formation, and while 40 mM imidazole was inhibitory, it is known to effect many other systems as well. Perhaps the use of sulfonamides to inhibit the enzyme will be more specific (Kannan, 1980). Understanding the role of carbonic anhydrase would be helpful in determining whether, CO_2 or HCO_3^+ is responsible for the dimorphic transition.

Another divalent cation which has recently been implicated in dimorphism is Ca^{++}. Roy and Gupta (1987) have recently reported that the calmodulin inhibitor trifluoperazine can block germ tube formation, as can the ionophore A23187. This latter effect can be reversed by Ca^{++}. Furthermore, they found an increase in the rate of protein phosphorylation during germ tube formation, which was completely lacking in growing yeast cells. Since calmodulin acts via protein phosphorylation both Ca^{++} and calmodulin were thought to play a role in dimorphism. Although, we never found Ca^{++} to be required for germ tube formation, or to specifically affect germ tube forma-

tion (it inhibited germ tube formation at the same concentration as other salts did), this may be because we never made an attempt to totally deplete all salts from the growth medium (except by using EDTA).

Of all the nutrient inducers, ethanol is probably the most enigmatic. We have shown that proline and GlcNAc serve as nutrients for germ tube elongation. This can readily be seen by the long germ tubes produced in their presence. The length of germ tubes induced by ethanol are not significantly longer than those induced by CO_2. It is difficult to argue that ethanol is a poor carbon source since Bell and Chaffin (1983) have shown that the density of cells grown on ethanol was no different than those grown on glucose. Thus, the possibility arises that germ tube formation induced by ethanol is different than proline- or GlcNAc-induction. One possibility is that ethanol somehow affects the solubility or hydration of CO_2 so that it is not readily lost from the medium at 37°C. It is, therefore, very likely that the study of ethanol-induced germ tube formation may also help explain the role of CO_2 in dimorphism.

The relationship of the utilization of the endogenous reserves to dimorphism needs to be reexamined. Gow and Gooday (1984) and Gow et al. (1986) have shown that vacuoles are formed on germ tube initiation, in all the different types of media known to form germ tubes. They proposed a model in which most of the cytoplasm of the initial germ tube is derived from the parent yeast cell. It is very likely, therefore, that endogenous reserves are extremely important in the initiation of germ tube formation. Very little is known about their utilization. Sullivan et al. (1983) showed that while trehalose degradation was not substantially different in germ tube forming and yeast growing cells, there was a difference in glycogen degradation by these

two cell types. However, using CO_2 as the inducer, and not supplying any exogenous nutrients, would be a superior method of looking at breakdown of endogenous reserves. Also, the breakdown may not be limited only to cytoplasmic reserves. Barrett-Bee and Hamilton (1984), Ram et al. (1984), Sullivan et al. (1984), and Molina et al. (1987) have all demonstrated the release of wall lytic enzymes by C. albicans. We have already discussed (Chap. 3) the possible relationship of proteases to dimorphism. Recently, Farley et al. (1984) demonstrated that vacuoles in C. albicans do contain proteases. Of possible significance in this context, is the report that CO_2 affects cell volume in Saccharomyces (Lumsden et al., 1987) and that mannitol catabolism is inhibited by glucose in Candida albicans. It is also interesting that only certain amino acids are capable of inducing proteinases (Fairbairn and Law, 1987).

With the use of CO_2-induction, the role of the cell wall in dimorphism may now be examined without the interference of the nutrient inducer. On the assumption that the cell will not synthesize products that are not needed, then, by forcing the cell to only use its endogenous reserves, it may be possible to carefully look at which polymers are needed for germ tube formation to occur. Thus, the role of the cell wall in dimorphism, may ultimately be confirmed.

SUMMARY

Factors affecting germ tube formation in Candida albicans were studied in order to elucidate some of the mechanisms of dimorphism in this fungus. Germ tube formation in C. albicans was found to be an endotrophic process, with no exogenous organic carbon or nitrogen being required to obtain germ tube growth. The sole exogenous chemical absolutely required for germ tube formation was carbon dioxide, which may be the actual inducer of germ tube formation. In addition, some new factors which affect germ tube formation, and the complex interrelationships between the many environmental factors which affect germ tube formation, were identified. This research showed that:

1. Ethanol induced germ tube formation.
2. Germ tube formation occurred at pH values between 3.0 to 9.0.
3. Glucose suppressed germ tube formation when the pH was below 5.5, but it stimulated elongation when the pH was above that value. Thus the reported pH-regulation of germ tube formation was disproved.
4. Germ tube formation could occur at temperatures as low as 22°C depending on the following factors: a) concentration of inducer; b) priming of cells; c) type of inducer; d) pH; e) presence of glucose; and f) presence of bicarbonate.
5. When bicarbonate was present during the final 4 h incubation at 25°C, along with an inducer, such as proline, only a short (30 min) pre-exposure to 37°C in buffered salts, was required for germ tube formation. Thus, the temperature and inducer requirements need not be simul-

taneous. Bicarbonate is also a hyphal growth factor.

6. Removal of the inducers, proline or N-acetylglucosamine (GlcNAc), following induction of germ tube formation, resulted in cessation of elongation.
7. There was no difference in the rate of uptake of either proline or GlcNAc from the induction medium under germ-tube-forming and non-germ-tube-forming conditions.

It was concluded that the nutrient inducers of germ tube formation (proline, GlcNAc, ethanol, and the combination of glucose and NH_4Cl), are most likely only serving as excellent carbon sources for the cells, and are probably capable of providing all the CO_2 required for germ tube formation at 37°C. Possible means whereby CO_2 might regulate germ tube formation are discussed. As the results suggested that the nutrient inducers must be metabolized in order for germ tube induction to proceed, the prior hypothesis that induction is gratuitous should be reevaluated.

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The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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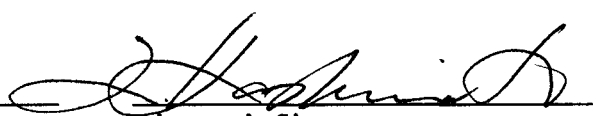
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