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GRADUATE COLLEGE

PHYSIOLOGICAL ASPECTS OF YEAST AND MYCELIAL FORMS OF *CANDIDA ALBICANS*

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

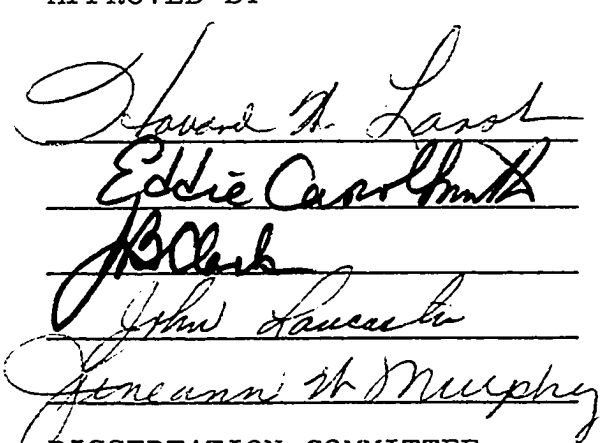
DENNIS STEVEN SCHWARTZ

Norman, Oklahoma

1979

PHYSIOLOGICAL ASPECTS OF YEAST AND MYCELIAL FORMS OF *CANDIDA ALBICANS*

APPROVED BY



DISSERTATION COMMITTEE

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ACKNOWLEDGEMENTS

Sincere thanks are due to all members of my dissertation committee for their help in the planning and execution of this work and for their constructive criticism of the product. Special thanks are due Drs. E. C. Smith and J. B. Clark for putting up with me all this time.

Deeply held feelings of gratitude are given to Dr. Howard W. Larsh through whose forebearance, great patience, faith and kindness I became a bit wiser as well as older and, perhaps, even somewhat more mature. Through words and deeds unspoken he pillowed my time during my darkest hours and allowed my life to always belong to me.

DEDICATION

This work is dedicated to the soft memory of my mother, Mary Schwartz. Her undying love and unfaltering faith in an inconstant son fostered a surge of self-belief and desire which overcame all encumbrances.

It is the saddest aspect of my life that she died before she could share with me the full joy of our mutual accomplishment.

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PHYSIOLOGICAL ASPECTS OF YEAST AND MYCELIAL FORMS OF *CANDIDA ALBICANS*

AN EXCELLENT MEDIUM FOR THE SELECTIVE GROWTH OF YEAST OR MYCELIAL FORMS OF *CANDIDA ALBICANS*: BIOCHEMICAL ASPECTS OF THE TWO FORMS

CHAPTER I

INTRODUCTION

Unlike several other pathogenic fungi, the dimorphism demonstrated by *Candida albicans* is determined by many factors. The literature abounds with reports of nutritional and environmental factors which favor the development and maintenance of one morphological form of *C. albicans* over another (9,13,27,38,41). Part of the difficulty in interpretation of these studies resides in that *Candida albicans* is, indeed, polymorphic. Many times different terms have been employed to denote identical events and vice versa.

The four distinct forms of *C. albicans* are: blastospore or yeast, chlamydospore, pseudomycelium and mycelium (50). For each of these forms there are developmental and transition stages. Primary interest has been the conversion

of yeast to mycelia which is the principle subject of this study.

Comparative studies necessitate the selective growth of one phase to the exclusion of another, with as few nutritional and environmental conditions altered as possible. Proper studies in physiology, biochemistry, immunology and pathogenics cannot adequately be performed without this ability.

All extant studies reflect the temporal and/or partial ability of the systems used to preferentially elicit the mycelial phase. Most recently, Lee et al. (24) detailed a synthetic defined medium in which C. albicans could be grown in either phase by manipulation of incubation temperature. As with most other studies however, they based their conclusions on results using only one strain of the organism. Having little success with this medium, we decided to investigate the possibility of developing a medium which would allow us to selectively induce and maintain the mycelial form of C. albicans. The medium we found suitable is a modification of a commercially available, autoclavable tissue culture medium.

A comprehensive, quantitative biochemical comparison of yeast and mycelia of C. albicans is also included in this study. This type of information is vital to the understanding of the metabolic differences existing between the two forms, allows for a more informed insight into the morphogenetic process, and points the way to further constructive research.

MATERIALS AND METHODS

All glassware was washed by the following procedure: soaked in H_2SO_4-dichromate cleaning solution for 24 hr at room temperature (22 C); rinsed 10X in tap water, 2X in double glass-distilled water (DDW); filled with DDW and allowed to stand at room temperature for 24 hr; washed in a laboratory washer (Heinicke), once with soap (7X, Flow Laboratories Inc.) and 3X without; placed in a hot air oven to dry.

Spectroscopy was performed with a Beckman model 24 double beam spectrophotometer with recorder using the appropriate blank in the reference cell as required.

A Bronson model 200 sonicator equipped with a micro tip attached to the standard horn assembly was used for sonication.

All biochemicals were from Eastman, other chemicals were of the highest quality commercially available.

Filter sterilization was done using Falcon #7103 units.

Organism, Inoculum, Media and Growth Conditions

Four strains of Candida albicans were employed in this study. All were clinically isolated at the Missouri State

Chest Hospital, Mt. Vernon, Missouri from cases of candidial vulvo-vaginitis and thrush. The identity of the organisms as C. albicans was confirmed through use of standard criteria: fermentation and assimilation patterns, germ tube production and formation of chlamydospores on corn meal agar (51). These strains were maintained on artificial culture medium (Brain Heart Infusion agar-Difco) for 3 months before our studies were initiated. The primary stock cultures had been transferred 3X in that period with storage at 4 C.

Inoculum was prepared through subculture onto several slopes of BHI agar. After incubation at 28 C and 70% relative humidity for 48 hr, the growth was washed from the slopes with 5 ml/tube of sterile 10^{-3} M K_2HPO_4-NaH_2PO_4/0.85% NaCl, pH 6.8 (phosphate buffered saline, PBS). The cell suspensions were decanted into sterile test tubes and centrifuged at 3,000 X g and 4 C for 10 min in an IEC-6000 centrifuge (IEC). The cells were then washed 5X in sterile PBS using alternate resuspension (vortexing) and centrifugation as previously described. Washed cells were suspended in 9 ml of sterile DDW to which 1 ml of 10X filter sterilized yeast nitrogen base (Difco) was added. The suspensions were maintained at room temperature for 6 hr with vortexing performed every hour followed by 12-18 hr at 4 C.

Just prior to inoculation, a viable count was determined by the procedure of Schwartz et al. (40) and each flask was inoculated with sufficient organisms to yield 10^6 colony-

forming units (CFU) per milliliter.

Sabourauds broth (Difco) was prepared according to package directions. The medium of Lee, Buckley and Campbell was made according to their article (24). Auto-Pow MEM (Flow Laboratories Inc., #1A-021 or #11-110-24) was modified as follows: the basic medium (10 g) was dissolved in 970 ml of DDW. To this solution 10 ml of 1 M K_2HPO_4-NaH_2PO_4 pH 6.8 was added. After thorough mixing, 10 ml of a 100X trace ion mixture (Difco manual-pp 252) which included $CoCl_2$ (400 ug/ml) was added. Solid glucose (9 g) and glycine (1 g) were added. The mixture was brought to 1 l with DDW and adjusted to pH 6.0 with NaOH. Before sterilization, all media were dispensed in 50 ml aliquots into 300 ml nefloflasks (Bellco #2574-12133). Sterilization was performed at 121 C and 15 psi for 15 min for the flasks containing Sabourauds broth, 110 C and 10 psi for 20 min for the flasks containing the medium of Lee et al. (24) and 112 C, 12 psi for 10 min for modified Auto-Pow MEM (AP+ medium). After cooling to room temperature, the flasks containing AP+ medium were brought to 0.05% N-acetyl-D-glucosamine (42) and 250 ug/l biotin through addition of a 100X filter-sterilized solution. The flasks were allowed to stand at room temperature for 24 hr as a check for contamination. Prior to inoculation, the flasks were pre-incubated for 4 hr at the conditions for growth of the mycelial phase. The conditions for the flasks with Sabourauds or the medium of Lee et al. (24) were 37 C

and 200 rpm in a New Brunswick Controlled Environment Shaker with a 1-inch stroke (psycrotherm). For the flasks with AP+ medium, the conditions were 40 C and 100 rpm in a psycrotherm.

Flasks were sampled every 3 hr for the first 18 hrs of growth and again at 24, 48, and 72 hrs post inoculation. Preparation of a sample for counting was performed through removal of a 2-5 ml portion of culture with a wide-mouth pipet which was delivered into a 50 ml disposable conical centrifuge tube. The volume was raised to 25 ml by addition of distilled water to which one drop of Tween 80 was added. The tube was then vortexed at medium-high speed for 15 sec at which time sonication was performed at a power setting of 5 for 10 sec (36). A sample was immediately removed with a Pasteur pipet and examined microscopically. At least 1000 CFU were counted at each representative data point. Yeast-mycelial ratios and distinguishing criterion were determined by the standard of Land et al. (23).

Preparation of Cells for Biochemical Testing

One strain (OU #1) of the four used in the previous procedures was employed in the following study. AP+ medium was prepared as described above and portioned in 500 ml aliquots into 2 l baffled flasks (Bellco #2543-02000). Yeast (Y) growth was elicited by incubation at 20 C and 200 rpm, mycelial (M) growth was obtained at 40 C and 100 rpm.

Cells were harvested during the period of most rapid, balanced growth (8,21). Yeast cells were harvested by first

pouring the broth through an ice-filled food strainer into an ice-containing 4 l beaker. Both the sudden temperature drop and the dilution of medium served to arrest metabolic activity (36). The growth was then collected by centrifugation at 7,000 X g and 4 C in a Sorvall SR-2B centrifuge. The supernatant was discarded and the pellet washed 5X with ice-cold PBS by alternate resuspension (vortex) and centrifugation as described above. The cells were then transferred to a 50 ml centrifuge tube (Bellco #3036-00050), resuspended in 3 ml of 1 mM $MgCl_2$, shell frozen at -70 C and lypholized on a Virtis (10-146-MR-BA) lypholizer (15). Upon drying, the sample was stored in a dessicator over anhydrous $CaSO_4$ at 4 C until used for biochemical determinations. Because of their buoyancy, mycelia could not easily be collected and washed by centrifugation. Cells were harvested by filtration through a Cistron sectional filter apparatus (Cistron Corporation #673) equipped with a 37 mu nylon screen filter. The collected growth was then washed 5X with 25 ml of ice-cold PBS. The resulting "plug" was removed, resuspended in 1 mM $MgCl_2$, shell frozen, lypholized and stored as before.

Cellular Biochemical Aspects

All biochemical tests were performed on duplicate samples with the mean value reported. Total cellular carbohydrate was determined by the phenol method (phenol-positive carbohydrate) (15). Total cellular protein was performed by biruet (15). DNA and RNA were determined through the following

procedure which is a combination of the assays found in Stewart (45). Samples (30-75 mg each) were delivered into test tubes. Rehydration was accomplished by the addition of 2-4 ml of ice-cold DDW, followed by the addition of an equal volume of ice-cold 0.5 M $HClO_4$. While incubating 15 min on ice, the samples were vortexed several times. The tubes were then centrifuged at 5,000 X g and 0 C for 10 min in the IEC and the supernatant discarded. This procedure was repeated once more with the cells recovered by centrifugation and the supernatant again discarded.

The samples were then resuspended in 4 ml of 0.5 M $HClO_4$ and incubated at 37 C in a water bath for 2 hrs with shaking every 15 min. The growth was pelleted by centrifugation as before and the extract withdrawn into a 10 ml volumetric flask. The cells were then washed with 4 ml of 0.5 M $HClO_4$, recentrifuged, and the wash combined with the first extract. The total volume was adjusted to 10 ml by addition of 0.5 M $HClO_4$ and assayed for RNA using the orcinol reagent.

The samples were resuspended in 3 ml of 0.5 M $HClO_4$ and heated at 70 C in a water bath for 15 min. The cells were pelleted by centrifugation as described above and the extract transferred to a 10 ml volumetric flask. This extraction and centrifugation was repeated twice and the extracts pooled. The samples were adjusted to 10 ml by addition of 0.5 M $HClO_4$ and analyzed for DNA using the diphenylamine assay (45).

Trehalose, alkali-soluble and acid-soluble glycogen, mannan and glucan were analyzed as described in Stewart (45). Chitin was determined by the following procedure: samples (300-800 mg each) were delivered into 50 ml sorvall centrifuge tubes (Sorvall #03149) and rehydrated by addition of 10 ml of ice-cold 50 mM Tris-HCl, 5 mM EDTA, 1 mM 2-beta mercaptoethanol (2-BME) buffer (pH 7.5). After 10 min on ice, the samples were mixed by vortexing and sonicated for 12 min at a power setting of 6 while cooled in a stirring ice-water bath. The samples were centrifuged at 10,000 X g and 4 C for 15 min in a Sorvall SR-2B centrifuge and the supernatants discarded. The crude cell wall pellet was washed 2X with ice-cold PBS through vortexing and centrifugation as above. The cell wall lipid was removed as outlined in Chattaway *et al*. (5). Ten ml of 4 N HCl was then added to each sample and hydrolized under N_2 for 12 hrs at 100 C (22). The samples were transferred to 25 ml volumetric flasks and brought to volume with DDW. Chitin (as free glucosamine) was determined as described in Strominger *et al*. (46).

RESULTS

The relative abilities of the tested media to induce and maintain the mycelial phase (M phase) of Candida albicans are presented in Figure 1. Results from all four strains used in this study were averaged and plotted with the standard deviations noted by error bars. The per cent mycelia in the medium of Lee et al. (24) steadily decreased through 15 hr at which point it stabilized around 5%. After 6 hr of incubation, no mycelial forms could be noted in Sabouraud's broth. AP+ medium, however, maintained mycelial growth up to 24 hrs. Even at 48 and 72 hrs post inoculation, the percentage of mycelia in AP+ was still very high (95%+). Macroscopic and microscopic views of yeast and mycelial forms of Candida albicans grown in AP+ are shown in Figures 2 and 3.

Results of biochemical tests and ratios of several cell components of the two forms of C. albicans are presented in Tables 1 and 2. All constituents are noted in ug/mg dry (lypholized) cell weight.

Figure 1. Figure is in the form of a superimposed histogram. Standard deviations are represented by error bars.  = AP+ medium;  = medium of Lee, Buckley and Campbell;  = Sabourauds broth

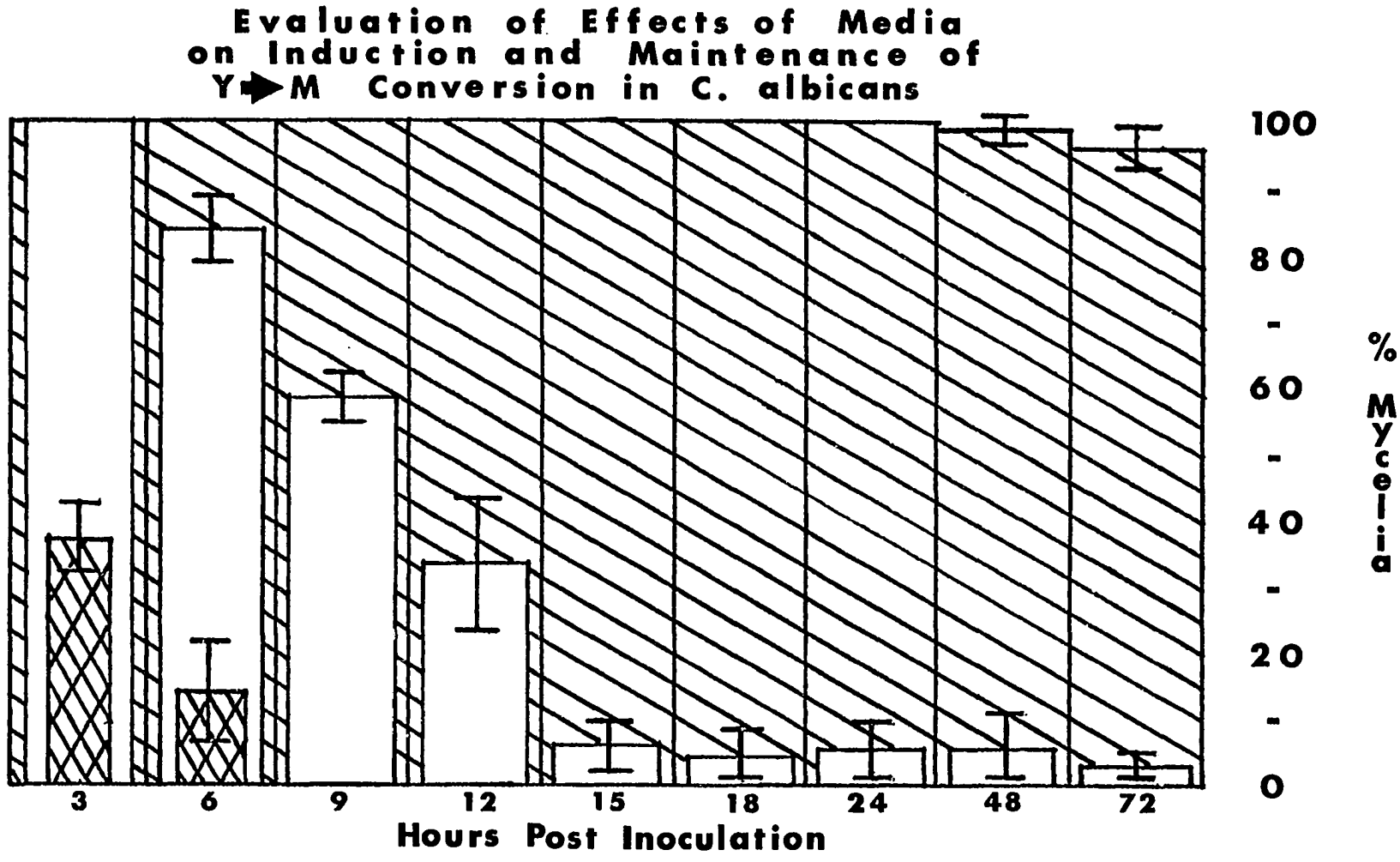

Evaluation of Effects of Media
on Induction and Maintenance of
Y→M Conversion in C. albicans
100
80
60
40
20
0
% Mycelia
3
6
9
12
15
18
24
48
72
Hours Post Inoculation

Figure 2. Macroscopic view of 24 hr cultures of the yeast and mycelial forms of Candida albicans elicited in AP+ medium. Yeast growth was at 20C and 200 rpm, mycelial growth was at 40C and 100 rpm. Growth is viewed in the necks of the employed nefloflasks.

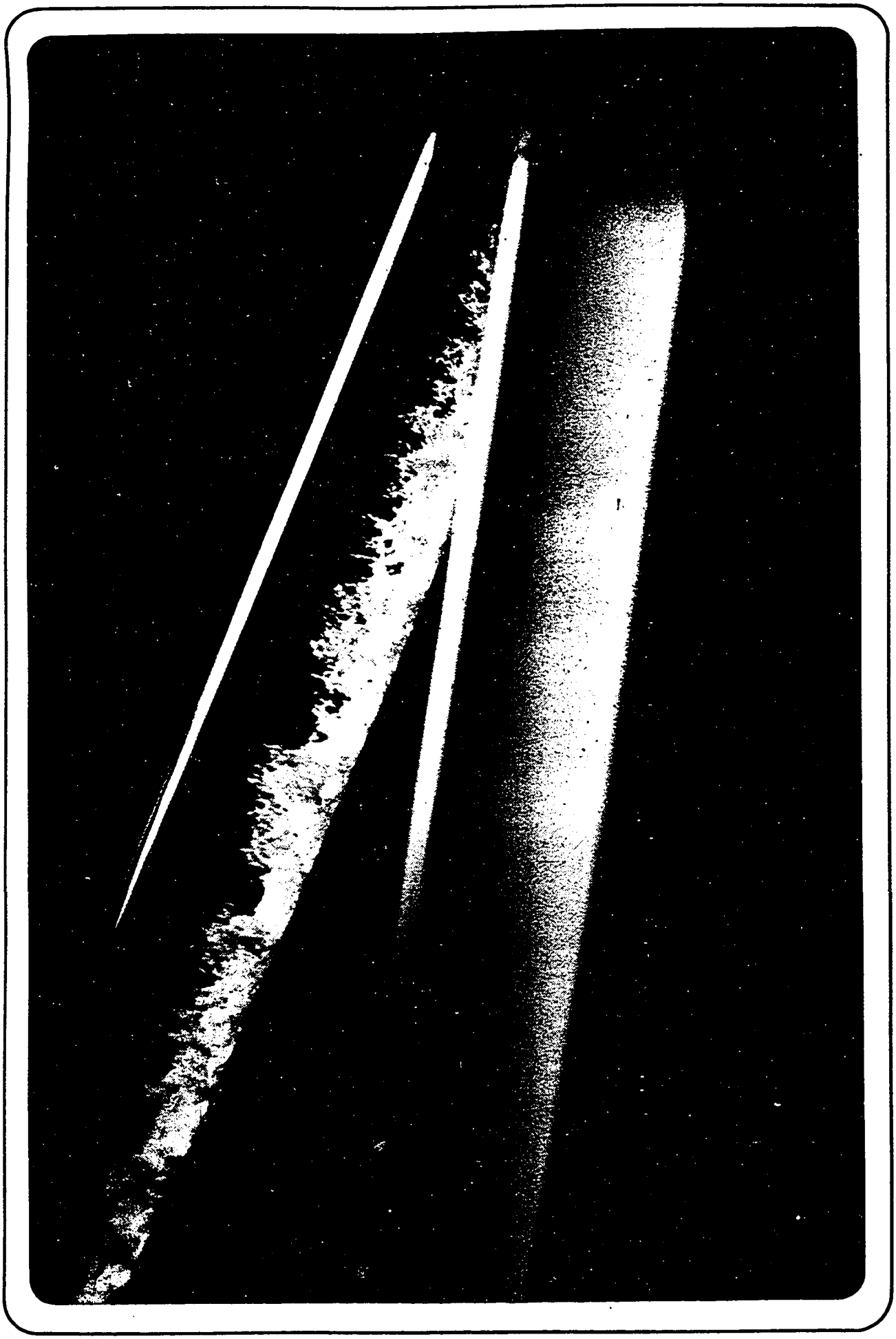
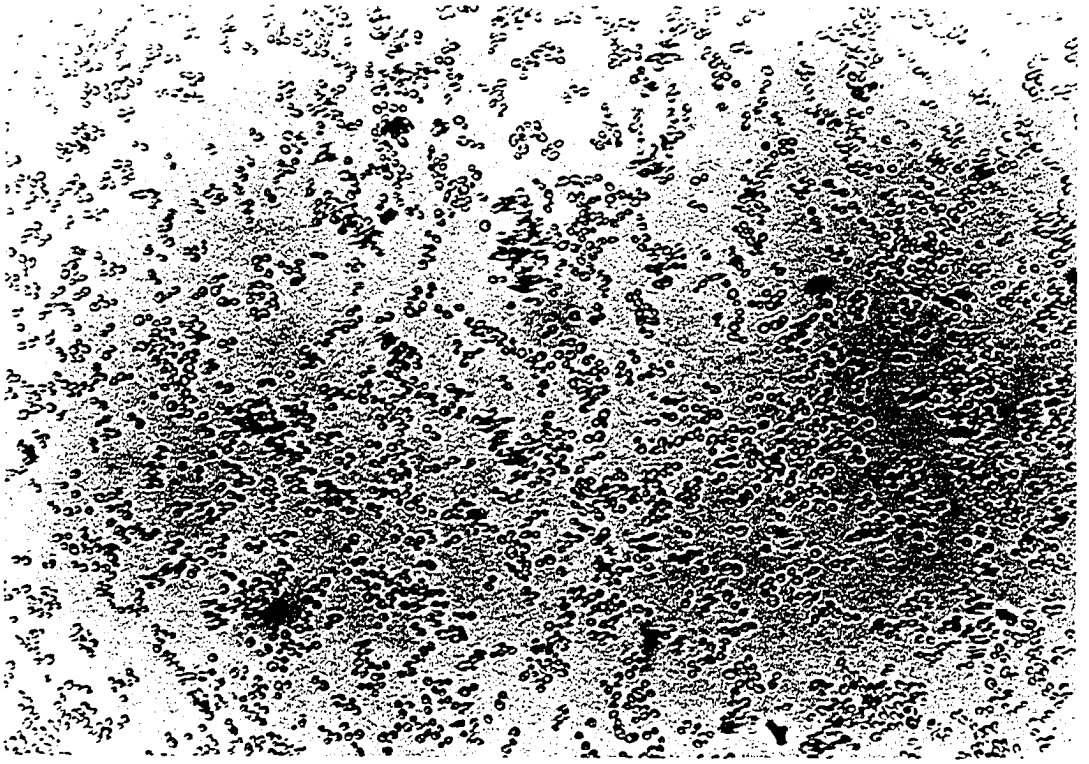


Figure 3 (a and b). a. (Top) Microscopic view (100 X) of log-phase yeast cells grown in AP+. b. (Bottom) Microscopic view of rapidly growing mycelia of C. albicans in AP+.



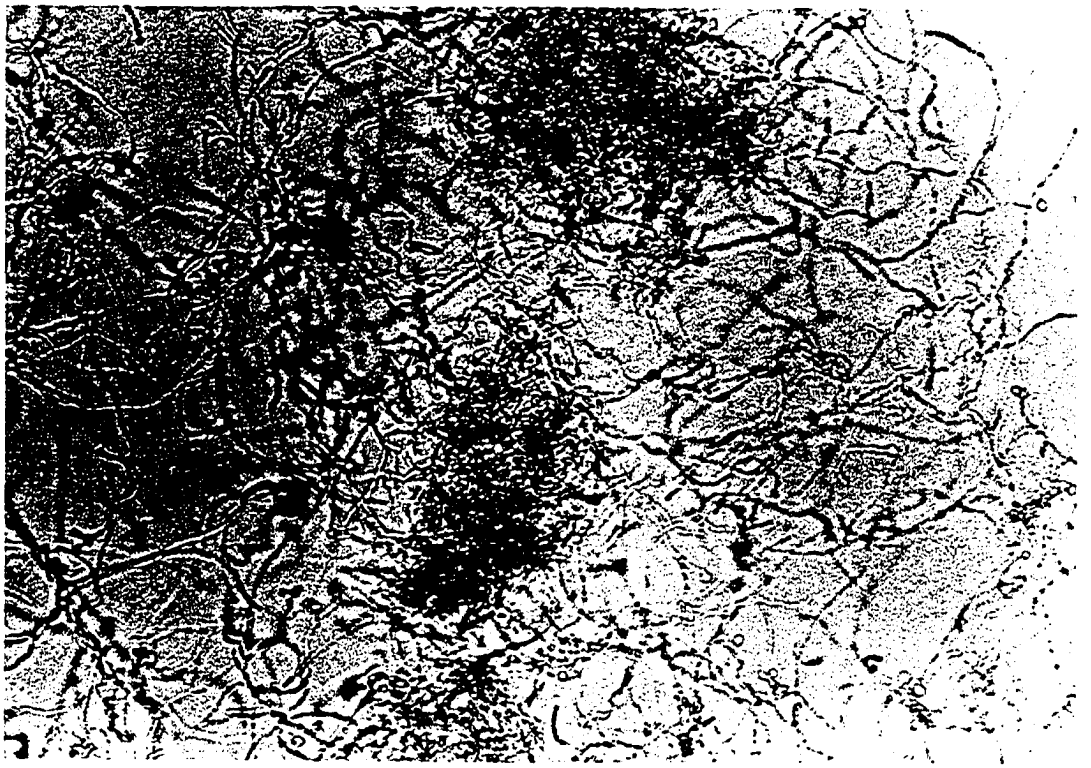


TABLE 1

BIOCHEMICAL COMPOSITION OF YEAST AND MYCELIA OF

CANDIDA ALBICANS GROWN IN AP+ MEDIUM

CELL CONSTITUENT	YEAST	MYCELIA
Total Protein	321[a,b]	348
Total Carbohydrate[c]	310	512
Trehalose	0	25.5
Glucan	63.03	76.50
Mannan	22.32	16.65
Alkali-Glycogen	51.13	52.28
Acid-Glycogen	42.3	80.5
Chitin	6.73	15.78
RNA	94.6	68.0
DNA	12.68	7.36

[a] Numerical values represent ug/mg dry (lypholized) cell wt.

[b] All determinations are averages of duplicate samples

[c] Total carbohydrate = phenol-positive carbohydrate

TABLE 2

RATIOS OF PRINCIPLE CELL COMPONENTS

COMPONENTS	YEAST	MYCELIA
Glucan/Mannan	2.82	4.59
Glucan+Mannan/Chitin	12.7	5.9
Chitin (M/Y)[a]	2.34	
Total Carbohydrate+Chitin (M/Y)	1.68	
$\frac{\text{Glucan+Mannan+Chitin}}{\text{Acid Glycogen}}$	2.18	1.35
Total Protein/RNA	3.39	5.12
$\frac{\text{Total Carbohydrate+Chitin}}{\text{Total Protein}}$	0.99	1.52
RNA/DNA	7.47	9.23
RNA+DNA/Total Carbohydrate+Chitin	32.7%[b]	20.7%
Alkali-Glycogen/Total Carbohydrate +Chitin	15.6%	9.91%

[a] (M/Y) = Mycelia amount/Yeast amount

[b] Presented as percentages for ease of comparison

DISCUSSION

It is very difficult to gather coherent information from the literature concerning the dimorphism of Candida albicans since much of it is contradictory and subject to serious interpretive scrutiny (9,41). Most existant studies employed only one strain of the organism and often the strain used was not a fresh clinical isolate but rather one which had been maintained on artificial media for a protracted time (23,28,29,31,33,52,53,54). Several of the most cited studies employed strains which had been genetically altered or physiologically selected for their performance in the particular test system (23,28,29,31,38).

The lack of standardization evident between different studies also lends confusion to data interpretations and comparisons. Lack of standardization is manifest in the misapplication of various terms used to describe the growth of C. albicans. The term "filamentation" has been used to indicate everything from germ tube production to true mycelial growth. Often, the terms pseudomycelium and mycelium are cited interchangably when they stand for two separate structures (30). It is my opinion that the term "filamentation" should be dropped and that more succinct term such as the

following be employed: germ tube production is associated with induction and therefore should be relagated to the first few hours of growth. Upon the production of septa a germ tube becomes a true mycelium which may later branch, form more true mycelium or pseudomycelium or give rise to secondary blastospores. Pseudomycelium can also develop directly from an induced blastospore (50). Short sonic treatment, such as employed in this study, might aid in distinguishing short pseudomycelial strands from clumps of blastospores which have not completed cytokenesis (36).

In almost all previous literature, it has been custom to count a clump or tuft of mycelia as one unit (CFU) since the enumeration of individual strands is extremely difficult. This procedure introduces large errors in counting which are not standardized from one data point to another. The sonication procedure outlined in materials and methods assists in obviating this error. Inocula prepared by various techniques, can lead to different growth manifestations. Except for studies whose procedures demand a direct transfer of cells from one growth condition to another, we suggest it is best to start with resting cells and an inoculum of from 2×10^5 to 10^6 CFU/ml (10,11,26,41,43). Selection of sized cells, if possible, would be a desirable refinement of procedure since it has been shown to be important for germ tube induction of C. albicans (4). An additional factor whose relationship to morphogenesis has not been examined is that C. albicans

exhibits autoploidization (47,48). Several of these polypoid states are, at least, temporarily stable. The conditions needed to induce and maintain these states are largely unknown.

Growth conditions which are obtainable in one system cannot always be duplicated in another. For example, the gas exchange and gradient nutrient depletion characteristics of surface growth on solid media is very different from the dynamics of submerged growth in shaken liquid culture. For organisms like C. albicans whose morphological variations are subject to wide environmental influence, it is not always possible to extend form determining aspects observed in one system to another.

In batch culture studies, an organism's physiology is also dependent on when it is harvested. Stationary phase cells can differ radically from mid-log cells. Standardization of this experimental aspect will do much to forward comparable data. For studies concerning active growth and reproduction of C. albicans, we suggest harvesting should occur during balanced growth (8,21).

The dimorphic mechanisms of most other pathogenic fungi do not exhibit the degree of form determining sensitivity to nutrition as C. albicans. For most strains, temperature alone serves to induce and maintain a particular form, almost irrespective of the nutritional conditions employed. With C. albicans, however, the form determining effects of each

nutritional and physical aspect is sensitively influenced by all others with the added complication that different strains are affected to a greater or lesser extent by any one condition. The result of this "balance of many factors" principle is manifested in the great number of experimental conditions which have been cited as eliciting morphogenesis in C. albicans (7,9,13,20,27,41). The above, together with previously mentioned considerations, leaves the various sets of outstanding data, to a large extent, incomparable (34).

It is likely that no one medium or set of conditions will work with all strains of C. albicans. However, in this study, AP+ medium elicited and maintained the Y and M forms of C. albicans much better than the one other medium advocated for this purpose (24). A divergent strain whose dimorphism was not well manipulated by AP+ medium could nevertheless, advantageously be studied. Through alteration of the chemical composition of the medium, and/or the physical aspects of growth, one or several characteristics might emerge as preeminent in determining form in the particular strain under study.

AP+ medium is easy to prepare, optically transparent and almost colorless (slight green tint), displays excellent anti-foaming characteristics and gives a good growth yield. Most importantly, AP+ is of known composition and is not subject to the variation encountered in batches of complex media. These variations in complex media also account for

some of the incomparability and irreproducibility of data with C. albicans (34).

The biochemical data indicates vast metabolic differences between the two forms. Chattaway et al. (5) noted differences in the cell wall composition of the Y and M forms of C. albicans, but since they employed a different fractionation scheme and used complex media, their results are not comparable with this study.

The carbohydrate parameters and their various ratios (Tables 1 and 2) indicate a much higher activity of carbohydrate utilization in the M form than in the Y form. Chitin was found to be appreciably higher in the M form, comparing reasonably well with those reported (5). Differences can likely be accounted for by the differences in growth conditions, harvest time and strain of organism used. The various ratios of cell wall components indicate considerable differences in tertiary structure (3,5,39). A manifestation of this diversity would be the demonstration of greatly differing immunological characteristics. Since the total amount of protein and phosphate present in fractions of wall material is also subject to alterations with strain and growth conditions (35) and since C. albicans can exist in many "transition" forms which display morphological characteristics intermediate to yeast and mycelium, the possible antigenic combinations complicate the problems of in vivo competence and in vitro testing. Additionally, it has recently been

suggested that acid-soluble glycogen is also involved in cell wall structure (14). The differing ratios of acid-soluble glycogen to other cell wall constituents of these two forms of C. albicans could provide further means for antigenic variation.

The absence of trehalose in the Y form, its presence in the M form, and the lower relative amount of alkali-soluble glycogen in the M form vs the Y indicate great differences in reserve carbohydrate metabolism. From the data it seems that glycogen metabolism is the only source of reserve carbohydrate in the Y form. However, in the M form the presence of trehalose metabolism indicates a diminished role for glycogen metabolism. The regulation of glycogen synthesis and breakdown is very complex involving many enzymes, several subject to allosteric and covalent modificational control, while trehalose metabolism is simply regulated by three enzymes (2). If the emphasis in the M form is on trehalose metabolism, this would indicate several things: (a) the M form is likely spending less energy than the Y form for operation of its reserve carbohydrate function with fewer enzymes needed to be synthesized. This energy is then available for other cell processes; (b) a shift to trehalose accumulation might also serve as a sensitive means to regulate hyphal osmotic pressure to prevent bursting or alterations in structure (18); (c) the much greater utilization of carbohydrate in the M form indicates a lower intracellular pool of glucose-6-phosphate

which has been shown to be one of the major stimulators of glycogen synthase in fungi (18); (d) since the biosynthesis of glycogen and trehalose have essentially the same precursors and since the M form of C. albicans is growing faster than the Y form (40 C vs 20 C) it seems that the demands for ATP would be higher in the M form than in the Y form. Presumably, this higher demand for ATP in the M form would lower the dynamic pool of ATP and raise the relative AMP level. This in turn would effect the activation of phosphorolytic degradation of glycogen and inhibit the hydrolysis of trehalose (44).

Insights from the nucleic acid data are difficult, especially when the role of autoploidization, if any, is unknown. However, several gross observations can still be made. The RNA/DNA ratios indicate a higher production (20 %) of RNA produced from DNA in the M form. Indeed, the 47 % lower amount of DNA in the M form points to vastly differing relationships between the major metabolic regulatory functions in the two forms of C. albicans. How much of the above can be viewed as a consequence of the contrasting nature of mycelial vs yeast growth is difficult to ascertain. Separation of cause and effect is not really available at this stage of investigation. While the above seems to beg the question, this author feels that no meaningful discussion of these various data can occur without more precise examination into the molecular biology of these

two forms of C. albicans under well-circumscribed, standardized conditions.

It is important to note that the biochemical results can only be correlated with the different forms of Candida albicans grown under the stated conditions; they cannot be causally related, nor are the presented metabolic speculations the only ones which could be considered. Indeed, because of the nature of the polymorphism of C. albicans, the described system is certainly not the only one which could be developed for the specific growth of the several forms of this organism.

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ELEMENTARY CARBOHYDRATE METABOLISM IN YEAST AND MYCELIAL FORMS OF *CANDIDA ALBICANS*

CHAPTER II

INTRODUCTION

The polymorphism demonstrated by *Candida albicans* is a many faceted phenomenon. Several studies have concentrated on eliciting the differential infective aspects of the several forms (18). For the most part, these studies have yielded inconclusive results. We regard this question as somewhat moot due to the rapid intraform convertibility of *C. albicans* and that cells grown under different conditions are very different physiologically (22). Unlike many bacteria and fungi whose very presence indicates disease, *C. albicans* is a common commensal whose virulence varies greatly between strains and whose pathogenicity can be strongly correlated with the overall physiology and health of the host.

Unlike several other fungal pathogens whose form is determined by alterations in incubation temperature, the polymorphism demonstrated by *C. albicans* is subject to sensitive influence by many factors. In any one culture system it is not at all unusual to find several forms

co-existing at a particular time, even though one form or another tends to predominate. A major difficulty in approaching the problem of morphogenesis in C. albicans has been the lack of an easily manipulable, defined system in which C. albicans could be exclusively grown in either of the two major forms, yeast (Y) or mycelial (M). The development of such a culture system is detailed in Chapter 1.

The study of morphogenesis in C. albicans is worthy for several reasons: (i) since the organism's morphogenetic events are gradual, somewhat sequential, capable of being synchronized and subject to fine manipulation with changes in the nutritional and/or physical environment, it would seem an ideal organism for which to study morphogenesis. Candida albicans (as well as several other members of the genus which demonstrate morphogenesis) appears to be a better tool for these types of studies than are other pathogenic, dimorphic fungi due to its rapid growth and ease of handling. (ii) the understanding of morphogenesis in C. albicans is very important to related problems in medical mycology--specifically, problems concerning the immunology and pathogenicity of this important fungal agent.

Of the many studies which have concentrated on the physiological differences between these two major forms of C. albicans, only two have dealt directly with the carbohydrate enzymology (2,29). These papers are not comparable and are open to serious interpretive question because of the

experimental methods employed. In lieu of these studies, we considered it worthwhile to investigate some of the enzymes of carbohydrate catabolism in the two forms of C. albicans especially since we had a very clean synthetic, defined system in which the organism could easily be manipulated.

MATERIALS AND METHODS

Procedures and equipment used in the treatment of glassware, filter sterilization, spectroscopy, sonication, preparation and portioning of inoculum, and conditions of culture and harvest of cells were performed as previously described in Chapter 1. The organism used in this study was Candida albicans strain OU #1 whose source and characteristics were described in Chapter 1.

Protein estimations were performed by the ultraviolet method of Kalb and Bernlohr (14). Enzymes, enzyme substrates and other biochemicals were from Sigma. Other chemicals were of the highest quality commercially available.

Auto-Pow MEM (Flow Laboratories #1A-021 or #11-110-24) as modified was prepared as previously described (AP+ medium). The use of this medium leads to reproducible, comparable results which are not subject to the variations encountered when complex media are employed (22).

All enzyme procedures were performed on crude extracts prepared by the following method: Freshly washed cells were transferred to a 50 ml centrifuge tube (Sorvall #1349) and resuspended in 30 ml of ice-cold, degased 50 mM Tris-HCl, 5 mM EDTA buffer, pH 7.5 (12). The samples were then adjusted

to 2 mM phenylmethylsulfonyl fluoride (PMSF) through dropwise addition of a 40 mM stock solution (24). Solid norite (powdered carbon) was added to a final concentration of 1% (w/v) (12). The samples were then mounted in a constantly stirring ice-water bath and allowed to cool for 5 min. Sonication was performed at the settings previously determined to elicit maximum protein release: for the yeast phase, 8 min at a power setting of 6; for the mycelial phase, 7 min at a power setting of 5. Sonication periods of two minutes were separated by 5 min rest periods to allow the mixture to cool. At no time did the sample temperatures exceed 14 C.

The sonicates were then centrifuged at 25,000 X g and 4 C for 30 min in a Sorvall SR-2B centrifuge (SR-2B). The supernatants were decanted into small beakers and the protein content determined. Protamine sulfate (2% solution) was delivered to the ice-chilled, slowly stirred samples to yield 7% of the total protein (25). The mixtures were stirred for an additional 10 min at which time they were recentrifuged as outlined above. The supernatants (@ 40 ml) were then placed in pre-treated dialysis bags (25) and dialyzed overnight at 4 C against 4 l of 50 mM Tris-HCl, 5 mM EDTA buffer, pH 7.5. Just prior to use, sufficient PMSF and Gentamyacin sulfate (Squibb) were added to the buffer to yield 2 mM and 5 ug/ml respectively (16,24).

These extracts were then assayed for protein and employed in the enzyme assays. Phosphoglucomutase, however,

has a serine residue at its active site and therefore, it was necessary to rid a portion of each sample of PMSF in order to assay for activity (17). This was accomplished by subjecting 10 ml portions to overnight re-dialysis at 4 C against 4 l of the above buffer without added PMSF. These samples were assayed for protein and then used in the phosphoglucomutase assay. The crude extracts were maintained at 0 C on ice during the test period.

NADP-dependent glucose-6-phosphate dehydrogenase (G-6-PDH) EC # 1.1.1.49, was assayed by the procedure of Domagk and Chilla (6). NAD-dependent alcohol dehydrogenase (ADH) EC # 1.1.1.1, was assayed as outlined in Klinman and Welsh (15). Hexokinase, EC # 2.7.1.1, was assayed by the method of Hirai et al. (11). Phosphoglucomatase (PGM) EC # 2.7.5.1, was assayed by the method of Daugherty et al. (4). Glyceraldehyde-3-phosphate dehydrogenase (G-3-PDH) EC # 1.2.1.12, was assayed by the procedure of Stallcup et al. (26), incorporating the modifications of Gennis (9). Phosphofructokinase (PFK), EC # 2.7.1.11, was assayed as outlined by Stellwagen and Wilgus (27). All enzyme kinetic data were analyzed using the direct linear plot of Eisenthal and Cornish-Bowden (7). Enzyme specific activities were computed as units of enzyme activity/mg protein. All results represent the mean value of duplicate determinations which never differed by more than 4%.

RESULTS

With the exception of phosphofructokinase (PFK), none of the homologous enzymes isolated from the two forms demonstrated differences greater than two-fold in K_m or V_{max} (Table 1a). Therefore, only the specific activities and their ratios are discussed (Tables 2a and 3a). Inter- and intraform ratios of specific activities serve as a crude but indicative comparative method of elicitating metabolic similarities and differences.

The K_m and V_{max} characteristics of PFK were as follows: for the Y phase; $K_m = 1.94 \times 10^{-2}$ mM, $V_{max} = 8.1 \times 10^{-2}$ uM/min for the M phase; $K_m = 8.5 \times 10^{-3}$ mM, $V_{max} = 3.2$ uM/min.

TABLE 1a

K_m AND V_{max} VALUES OF SOME PRINCIPLE CARBOHYDRATE CATABOLIC ENZYMES IN THE Y AND M FORMS OF CANDIDA ALBICANS

ENZYME	Y[a]		M	
	K_m	V_{max}	K_m	V_{max}
HEXOKINASE	1.17×10^{-1}[b]	6.66×10^{-2}[c]	2.5×10^{-1}	6.5×10^{-2}
G-3-PDH[d]	2.2×10^{-1}	4.45×10^{-2}	1.08×10^{-1}	3.74×10^{-2}
PFK[e]	1.94×10^{-2}	8.1×10^{-2}	8.5×10^{-3}	3.20
PGM[f]	3.87×10^{-2}	3.79×10^{-2}	NIL	
G-6-PDH[g]	2.37×10^{-2}	1.69×10^{-2}	4.6×10^{-2}	3.2×10^{-2}
ADH[h]	NIL		NIL	

[a] = Yeast form, mycelial form; Y and M

[b] = K_m values are presented as mM

[c] = V_{max} values are presented as um/min

[d] = glyceraldehyde-3-phosphate dehydrogenase

[e] = phosphofructokinase

[f] = phosphoglucomutase

[g] = glucose-6-phosphate dehydrogenase

[h] = alcohol dehydrogenase

TABLE 2a

SPECIFIC ACTIVITIES OF SOME PRINCIPLE CARBOHYDRATE CATABOLIC ENZYMES IN THE Y AND M FORMS OF CANDIDA ALBICANS

ENZYME	Y[a]	M
HEXOKINASE	0.669	1.16
G-3PDH[b]	0.487	0.342
PFK[c]	0.606	0.892
PGM[d]	0.402	NIL
G-6-PDH[e]	0.684	0.364
ADH[f]	NIL	NIL

[a] = Yeast & Mycelia (Y & M)

[b] = glyceraldehyde-3-phosphate dehydrogenase

[c] = phosphofructokinase

[d] = phosphoglucomutase

[e] = glucose-6-phosphate dehydrogenase

[f] = alcohol dehydrogenase

TABLE 3a

RATIOS OF SPECIFIC ACTIVITIES OF PRINCIPLE ENZYMES IN THE Y AND M FORMS OF CANDIDA ALBICANS

ENZYMES	Y[a]	M
G-6-PDH/HEXOKINASE	1.02	0.314
PFK/HEXOKINASE	0.906	0.769
PFK/G-3-PDH	1.24	2.61
G-6-PDH (Y/M)[b]	1.88	
HEXOKINASE (Y/M)	0.58	
PFK (Y/M)	0.68	

[a] = all notations in table 2a apply here

[b] = ratio of activity; yeast form/mycelial form

DISCUSSION

The most pronounced feature of the data is the significant activity of PGM in the Y phase and its absence in the M phase. This datum correlates well with observations made in Chapter 1 which demonstrated the presence of trehalose in the M phase of C. albicans and its absence in the Y phase. These data indicate that under the stated conditions of growth and harvest, there seems to be a shutdown of cytosol glycogen turnover in the M phase while in the Y phase glycogen metabolism is still very active. This conclusion, however, does not preclude active glycogen metabolism (acid soluble glycogen) associated with the cell wall in the M phase as inferred from the work of Gunja-Smith et al. (10).

Hexokinase activity in the Y form is only 58% of that noted in the M form. This indicates a much higher utilization-incorporation of glucose in the M form. This difference is made manifest by the observed absence of PGM activity in the M form. The increased hexokinase activity in the M form can be partially viewed as a response for the increased need of the organism for cell wall protomers. The above point is substantiated by the data in Chapter 1 which shows a greater amount of hexose sugars in the M form than in the

Y form. These observations do not support Nickerson's speculations into the morphogenetic processes in C. albicans since hexokinase is far more active in the M phase and that ample carbohydrate was available for the growth of both forms (20).

The specific activities of hexokinase and G-6-PDH and their ratios indicates that a greater percentage of glucose, phosphorylated by hexokinase, proceeds via the HMP pathway in yeast than in mycelia. The comparative activities of G-6-PDH is of special note as its activity, indicative of the activity of the HMP pathway, has received much attention as a key determinator of morphogenesis in fungi (1,3,28). Since C. albicans has demonstrated the ability to oxidatively form ribose and since the overall rate of formation of NADPH is controlled by the cellular level of the reduced co-enzyme, it follows that the demand for NADPH for biosynthetic reactions (the formation of unsaturated fatty acids and sterols for membrane synthesis) is higher in the Y form than in the M form. Most studies on the dimorphism of Candida albicans have used a temperature differential; yeast are grown at a lower temperature than mycelia. Therefore, the demand for unsaturated fatty acids would be higher in the yeast form (19,21,28). Presumably, the greater the temperature differential employed in selectively eliciting the two forms, the greater would likely be the differences in amounts of unsaturated fatty acids demonstrated in the two

forms (3,21). Even when grown at the same temperature in a culture system which yields both forms of the organism, yeasts of C. albicans contain more total lipid than mycelial cells. (19). This indicates that the production of NADPH would likely be higher in yeast even when grown at the identical temperature as mycelia. Slight modification of the fatty acid metabolism through modulation of key NADPH-producing enzymes of the HMP pathway has demonstrated a dramatic effect upon the morphology of fungi (28). The physical and chemical nature of the plasma membrane could, presumably, dictate cell wall biogenesis because of its direct involvement with the secretory and synthetic processes. Alteration of fatty acid content of plasma membranes could serve to radically alter the activity of certain cell-membrane associated enzymes (adenyl cyclase) whose operations have far reaching effects on the overall physiology of the cell, and perhaps directly effect the rate and mode of cell wall synthesis (28).

It has been very popular to relate this observed enhancement of NADPH production in the Y form with the process of cell division linked through an FAD^+ (diaphorase) center. Supposedly, this reducing power is then transferred to the cell division process through action on disulfide bonds mediated by a disulfide reductase enzyme (2,20). The major manifestation of these works leads to a particular mode of thought concerning morphogenesis in C. albicans which characterizes mycelial growth as an abnormal arrest of "normal"

cell division processes (20). Action on disulfide bonds has been demonstrated to be involved in blastospore reproduction in C. albicans. However, in light of this study and those of other recent workers, their importance in morphogenesis seems to be vastly overstated (8,13,28,30).

The ability of C. albicans to form germ tubes in serum and characteristic mycelium/pseudomycelium on certain laboratory media are taxonomic criteria. If a strain did not demonstrate these abilities it would be regarded as aberrant or not as C. albicans at all. Knowing this, and that the morphology of C. albicans is subject to gross alteration with slight modulation of the nutritional and/or physical environment, it is this author's opinion that the potential for morphogenetic transition(s) is inherent to the organism and should be viewed as a normal teleonomic reaction to changes in its immediate surroundings.

PFK is 47% more active in the M form than in the Y form. This aspect by itself might lead to the conclusion that glycolysis is much more active in the M form. However, when the data on PFK are standardized by comparison with those for G-3-PDH and hexokinase, significant metabolic relationships can be inferred. Since the activity of G-3-PDH is 42% higher in the Y form and since the ratio of PFK to G-3-PDH is much higher in the M form, it would appear that much more of the product of PFK goes onto the second half reactions of glycolysis in the Y form than in the M form.

The increased activity of the reactions of the first half of glycolysis noted in the M form is reinforced by noting the absence of PGM activity and the lower activity of G-6-PDH. The above characteristics would tend to push more substrate along the first steps of the EM pathway. In the M form, the first half products along the EM pathway would, as mentioned before, be drawn off for the presumed increased cell wall synthesis and for the likely increased shunting off of dihydroxyacetone phosphate (created by the action of aldolase, the next enzyme after PFK in the glycolytic sequence) to glycerol production via sn-glycerol-3-PDH. Since the M form is growing much faster at 40 C than the Y form at 20 C, this presumed enhancement of glycerol production, leading to increased lipid for cell membrane synthesis, would seem likely. The higher activity of PFK and hexokinase in the M form can also be viewed as indicative of a lower dynamic pool of ATP. If this is found to be the case, then this data could serve to substantiate the proposed reserve carbohydrate scenario put forward in Chapter 1. The comparative K_m and V_{max} for PFK in the two forms also indicate how much more active the enzyme is in the M form. One cautious note concerning the above scenario is that the amount of glyceraldehyde-3-phosphate produced in the Y form due to the increased activity of the HMP pathway (as judged by the higher activity of G-6-PDH in the Y form) would, most likely, be greater than in the M form. What contribution this source

of substrate has on the activity of G-3-PDH in the two forms of Candida albicans is unknown.

The total absence of ADH activity in both forms of the organism indicates that metabolism in C. albicans is aerobically manifested and that neither form, at least during log-phase, balanced growth conditions, demonstrate a crabtree effect such as that shown by Saccharomyces cerevisiae (5).

The indicated metabolic characteristics need to be substantiated through much more complete investigations of the various energy pathways (e.g. TCA, Glyoxylate) in the two forms of C. albicans. However, the yeast form's apparent higher energy requirement is directly inferred from the data and can be substantiated by several biochemical characteristics: as discussed previously, the Y form has need of increased reducing power (primarily in the form of NADPH) for the presumed higher production of sterols and unsaturated fatty acids. Additionally, since each yeast cell is an independent reproductive unit, a large amount of energy must be allocated for the synthesis of an entire genome and set of genome-related organells each time a "unit" of protoplasm is reproduced. This is not the case for the M form as the biochemical data in Chapter 1 indicates.

It is very difficult to relate findings in this study to those of other workers as these other studies employed vastly different and somewhat unorthodox methods (2,29). The

results of this study do stand in direct opposition to those of Ton *et al.* (29). These workers noted no differences in carbohydrate enzyme activity in these two major forms of *C. albicans*.

The presented hypotheses conform well to the data. However, it should be recognized that, especially with enzymes such as PFK and G-3-PDH, *in vivo* regulation of enzyme activity is sometimes subject to fine allosteric and/or covalent modificational control, effects of which are not discernible in this type of study. Additionally, interpretation of enzyme data is subject to pitfalls which can only be obviated by more thorough study and use of other available investigative techniques (3,28).

This study derives much of its substantiation from the use of a synthetic, defined media and set of growth and harvest conditions in which the two major forms of *C. albicans* were exclusively fostered. Other studies into the carbohydrate catabolic enzymes of *C. albicans* were performed on cells from complex systems whose demonstrated ability to maintain pure growth of the M form was poor (2,29). In order for future work to be comparable and reproducible, *C. albicans* should be grown and harvested under standardized conditions in a system which is as synthetic and defined as possible.

SUMMARY

A new synthetic, defined medium which allows for the exclusive growth of yeast or mycelial forms of Candida albicans is described. This medium is based on a modification of a commercially available, autoclavable tissue culture medium and is quick and easy to prepare. The medium demonstrated superior ability over the one other extant medium cited for the induction and maintainence of these two forms of Candida albicans.

Through use of this medium, the basic biochemical makeup and activity of several major carbohydrate catabolic enzymes in these two forms of Candida albicans were examined. The results indicate vast metabolic differences between the two forms. These data are discussed in relationship to the phenomenon of morphogenesis in C. albicans, which in turn, relates to problems in immunology and pathogenics of this important fungal agent.

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