



Nuclear magnetic resonance spectroscopy and cyclic AMP studies of *Candida albicans*
by Pamela Jane Purus

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE
in MICROBIOLOGY

Montana State University

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Abstract:

Candida albicans is a polymorphic and opportunistic fungus. As a saprobe *C. albicans* exists in the yeast (Y) form. Upon tissue invasion, mycelial (M) forms are produced. Cyclic AMP is one of many factors that have been studied as possible initiation factors in morphogenesis. However the methods used have involved environmental parameters that do not reflect the in vivo situation. Through the presence of an inhibitor of morphogenesis, morphogenic autoregulatory substance (MARS) produced by Y form cells the effects of cAMP were reevaluated by a system that more closely reflects the in vivo situation. In comparing levels of cAMP, no difference was found between the M and Y forms at the early time periods. There was an increase in the M form over the Y after morphogenesis was completed. These data indicate that cAMP is probably not important as an initiation factor in morphogenesis. Dimorphism assays are usually cell destructive techniques. Nuclear magnetic resonance (NMR) spectroscopy is an alternative method that allows live cells to be studied without disrupting cellular integrity. The Y and M forms were found to be distinguishable from each other by NMR. Spectra obtained from 10 min incubated cells was different for the M and Y forms. However identification of specific peak differences was not possible because of reproducibility problems encountered. Therefore base line studies of the NMR spectra of yeasts were begun. *C. albicans*, *C. tropicalis*, *C. krusei*, *C. guilliermondii*, *C. pseudotropicalis* and *Torulopsis glabrata* were used in an attempt to identify yeasts by their NMR spectra. Each of the species resulted in different NMR spectra that were reproducible. Twenty four *C. albicans* strains were grown under the same standard conditions. Three spectral types were noted, but no identification of strains was possible. Base line studies were done on *C. albicans* in order to determine what changes in growth conditions that affect the cells will change the NMR spectra. Changes in media, temperature of incubation, and growth phase of the organism change the NMR spectra. There were also effects on the cells and therefore the spectra due to continuous passage, mouse passage and mixed culture of the *C. albicans* cells. Variables involved in the growth of cells must be studied thoroughly before continuing with this complex problem.

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ABSTRACT

Candida albicans is a polymorphic and opportunistic fungus. As a saprobe C. albicans exists in the yeast (Y) form. Upon tissue invasion, mycelial (M) forms are produced. Cyclic AMP is one of many factors that have been studied as possible initiation factors in morphogenesis. However the methods used have involved environmental parameters that do not reflect the in vivo situation. Through the presence of an inhibitor of morphogenesis, morphogenic autoregulatory substance (MARS) produced by Y form cells the effects of cAMP were reevaluated by a system that more closely reflects the in vivo situation. In comparing levels of cAMP, no difference was found between the M and Y forms at the early time periods. There was an increase in the M form over the Y after morphogenesis was completed. These data indicate that cAMP is probably not important as an initiation factor in morphogenesis. Dimorphism assays are usually cell destructive techniques. Nuclear magnetic resonance (NMR) spectroscopy is an alternative method that allows live cells to be studied with out disrupting cellular integrity. The Y and M forms were found to be distinguishable from each other by NMR. Spectra obtained from 10 min incubated cells was different for the M and Y forms. However identification of specific peak differences was not possible because of reproducibility problems encountered. Therefore base line studies of the NMR spectra of yeasts were begun. C. albicans, C. tropicalis, C. krusei, C. guilliermondii, C. pseudotropicalis and Torulopsis glabrata were used in an attempt to identify yeasts by their NMR spectra. Each of the species resulted in different NMR spectra that were reproducible. Twenty four C. albicans strains were grown under the same standard conditions. Three spectral types were noted, but no identification of strains was possible. Base line studies were done on C. albicans in order to determine what changes in growth conditions that affect the cells will change the NMR spectra. Changes in media, temperature of incubation, and growth phase of the organism change the NMR spectra. There were also effects on the cells and therefore the spectra due to continuous passage, mouse passage and mixed culture of the C. albicans cells. Variables involved in the growth of cells must be studied throughly before continuing with this complex problem.

INTRODUCTION

Candida albicans is a normal inhabitant of the mucocutaneous regions of the human oral cavity, upper respiratory tract and intestinal tract (23, 59). This endogenous species of yeast usually does not produce disease in healthy individuals. However, through alteration of host innate or acquired immune mechanisms, an opportunistic infection may occur. Many factors can compromise the host and possibly lead to disease. Among these factors are: 1)endocrine dysfunction; 2)physiologic changes such as pregnancy or early infancy; 3)prolonged antimicrobial therapy; 4)immune deficiencies; and 5)iatrogenic factors like indwelling catheters and surgical procedures (37, 59, 78). The range of diseases caused by Candida spp. is collectively called candidiasis. Candidiasis can vary from acute to chronic, superficial to disseminated disease (23, 59).

The most common etiologic agent of candidiasis is Candida albicans. C. albicans is a polymorphic fungus. As a saprobe, the organism exists as a budding yeast. However, during tissue invasion, it undergoes morphologic change to form hyphae, pseudohyphae and yeast cells (34). Other dimorphic fungi such as Histoplasma capsulatum, Blastomyces dermatitidis and Sporothrix schenckii have been studied and morphogenesis has been

shown to be an important link to pathogenesis (59). In these latter cases the tissue form is the yeast form and the saprobic form is a mould.

The presence of hyphae and yeast cells in tissue of candidiasis patients has led to a controversy over which is the important form for pathogenesis. Investigators have shown that with natural and experimental infections the lesions may consist only of the yeast (Y) form (57, 79). However most investigators have implicated the mycelial (M) form in pathogenicity, perhaps due to it's ability to escape phagocytosis (19, 20, 43).

Studies on the polymorphic nature (or dimorphism) of Candida albicans were primarily directed towards surveying factors in vitro for their effect on morphogenesis. Some factors, for example proline (16, 17, 40) and zinc (4, 68, 81), when added directly to growing cultures promote an increase or decrease in Y-->M conversion. Intracellular levels of substances such as cyclic AMP (14, 53) and activities of enzymes like N-acetylglucosamine kinase (5, 62) and phosphofructokinase (12, 61) have been looked at in both the Y and M forms and differences in concentrations or activities have been noted.

The studies on dimorphism in C. albicans were done by many researchers in various labs, using different strains of organisms, types of media, methods of Y-->M conversion, protocols for cell breakage, extraction of the cellular products, and enzyme assay methods. This has led to conflicting data and confusion over the question of the role of specific factors in dimorphism. For example N-acetyl-D-glucosamine is one of many factors that have been implicated by different researchers to both promote (36, 49, 66, 69) and inhibit (77) Y-->M conversion. In an effort to dispel some of the confusion surrounding dimorphism a standard system that perhaps more closely reflects the in vivo situation was used to reevaluate an extensively studied factor, cyclic AMP.

Cyclic adenosine 3',5'-monophosphate (cAMP) has been studied as a possible factor in intracellular regulation of fungi (54, 73, 74). cAMP has been shown to be involved in the development of the cellular slime mould, Dictyostelium discoideum (80) and is implicated as important in the Y-->M form conversion in Histoplasma capsulatum. In H. capsulatum, it was found that the level of cAMP was approximately five times higher in the M form than the Y form, and that addition of dibutryl cAMP resulted in a Y-->M conversion (47, 51).

Work with the dimorphic fungus Mucor racemosus, by Sypherd et al (41, 55) indicated the reverse situation with Mucor. Intracellular levels of cAMP increased dramatically in the M-->Y conversion, before any visual signs of morphologic change. The level of cAMP reached a maximum at the time of conversion and dropped back to normal levels during exponential growth of the organism indicating that cAMP may be important in initiation of morphogenesis.

cAMP levels in C. albicans have been studied by other groups of researchers. Both Niimi et al. (53) and Chattaway et al. (14) reported that the Y-->M conversion in C. albicans was accompanied by a rise in cAMP. Chattaway et al. (14) reported that the increase in cAMP only occurred when early stationary phase (18 h) 30°C grown Y cells underwent a Y-->M conversion under the following conditions. The conditions were a temperature shift to 37°C, nutritional shift from Sabouraud broth with glucose to a starch, yeast extract, peptone broth (11) and the addition of partially purified serum protein (13). Niimi et al. (53) used early stationary phase (18 h) 37°C grown cells shifted to 30°C to induce the Y form cells and a shift to 40°C to achieve Y-->M conversion or M form cells. Niimi's group (53) noted a higher level of cAMP in M vs Y form cells using this system of conversion

in either of 2 different media.

A major problem with the work of Niimi (53), Chattaway (14) and other researchers, is the use of temperature shifts to induce morphogenesis. Many investigators maintain Y form cells by growing them at 28-30°C. To achieve a Y-->M conversion, cells are shifted from 28 or 30°C to 37 or 40°C. This shift in temperature probably does not reflect what happens during a natural progression from commensalism to disease. C. albicans as a saprobe or member of the normal flora grows at 37°C as Y cells on the surface of mucosal membranes. Thus, a temperature shift does not occur upon invasion of tissue and formation of hyphal forms.

A better way to induce the Y-->M conversion may be the use of morphogenic autoregulatory substance (MARS) produced by the Y form cells of C. albicans (30, 31, 32). This substance promotes cell replication but suppresses germination (Y-->M conversion) under conditions that would normally promote germination. Through the use of MARS both M and Y forms can be studied at the temperature of 37°C, using the same medium with only the addition of MARS to suppress germination.

Another major problem in research on dimorphism in C. albicans is the 2 different approaches used in

studying dimorphism. Many investigators have studied the M and Y forms after completion of morphogenesis (52). These studies were done to determine differences in antigens (46, 71), cytoplasmic proteins (6, 8, 45) and cell wall compositions (11, 22, 70) of the 2 distinct M and Y forms. However, an alternative approach is to study the cells prior to a change in morphology and look at factors that may play a role in initiation of this morphogenic process. Problems arise when investigators, studying cells that have completed the morphologic change, try to make conclusions about initiation events. An example of this is the work of Niimi, et al. (53) and Chattaway, et al. (14) who report a rise in cAMP at the time of morphologic evidence and assume that cAMP was involved in initiation.

The studies described so far were done by traditional biochemical methods. For these investigations cells were broken open. The resulting cellular contents were studied through biochemical analyses for enzyme levels, RNA concentrations and concentrations of pathway intermediates. This process disrupts cellular integrity and compartmentalization. Membranes become broken and it should be expected that control substances of enzyme activity, such as allosteric effectors, are diluted away. This would lead to results that do not reflect the levels

within the cell. A technique that is not invasive and would therefore reflect the intracellular situation would be preferable.

Nuclear magnetic resonance (NMR) is an alternative method which allows subcellular analyses without disruption of cellular integrity. This form of spectroscopy relies on the existence of a magnetic moment in some isotopes of atomic nuclei. When placed in a stationary magnetic field these nuclei behave like tiny bar magnets, aligning with or against the field. When the nuclei in the magnetic field are pulsed with a range of radiofrequencies the nuclei absorb energy of a specific frequency that is dependent upon the chemical environment of the atoms of interest. Each chemically unique nucleus will therefore absorb energy of a certain resonant frequency. Once pulsed the nuclei are allowed to decay and the emission of the absorbed energy results in a free induction decay (FID) which is then Fourier transformed. The result is a spectrum of different energy shifts compared to a standard. This spectrum can be directly related to the atoms of the different compounds.

In living systems, the NMR nuclei most commonly examined are ^{31}P , ^1H and ^{13}C (60, 64, 65). There are 2 ways to use these nuclei to study living systems through

NMR. The first method is called natural abundance NMR because the NMR isotopes used are those that naturally occur within cells. There is a limited sensitivity however and only high concentrations (0.01 M), of low molecular weight compounds will be seen. ^{31}P accounts for nearly 100% of all phosphorus found in natural occurrence and therefore is readily detectable in living systems by natural abundance NMR. ^{31}P has been used to look at phosphorus pools in both cell extracts (26, 38, 39) and intact organisms (2, 7, 25). ^{13}C has also been used in natural abundance work (39, 48, 72) although not to the full potential because of the low natural abundance of ^{13}C nuclei.

The second method involves the addition of labelled compounds to metabolizing cells. ^{13}C is ideal for this work because it has a low natural abundance of only 1.1 %. This allows the cell sample to be enriched with a ^{13}C labelled compound and there will be very low natural background of ^{13}C . The metabolism of the labelled compound can be followed by following the changing peak heights of intermediary metabolites in a spectrum over time (3, 18, 21, 26).

^{31}P NMR has been used to study dimorphism in C. albicans by Cassone et al. (9). Cassone et al. (9) looked at the Y-->M form conversion in C. albicans by using many

of the same methods used by Niimi et al. (53), Chattaway et al. (14) and others. The Y form cells were grown in a nondefined (yeast-extract, glucose) medium at 28°C and incubated for 8, 40 or 48 hours before NMR analysis. The Y-->M conversion was achieved by inoculating 24 h Y form cells into a second medium at the higher temperature of 37°C. M form cells were harvested at 90 and 240 min. for NMR analysis. Although the use of temperature and nutritional shifts are standard procedure in most laboratories for Y-->M form conversion, these procedures may lead to uncertain results when using the NMR approach. The differences in spectra obtained due to changes in either temperature, media or growth phase of organism can be drastic. Also the use of chemically defined media is important for reproducibility of spectra and attempts at identifying intracellular compounds, because of the known and consistent ingredients in defined media.

A Y-->M conversion system that does not depend upon a temperature shift (ie., all growth at 37°C) and utilizes a defined medium, would reduce the variables encountered by Cassone, et al. (9). In addition, the use of MARS as a germination suppressor would control for the effect of the nutritional shifts. However, in working out

a system of Y-->M conversion that reflected the in vivo situation and was compatible with NMR, another group of variables was encountered. Dimorphism is not a simple biological event in which the Y form converts into 100% true hyphae. A number of intermediate forms, apparently each with their own biochemistry, form during a Y-->M conversion. This results in a multitude of spectra due to the varying phenotypes.

Along with this problem is the strain variation of C. albicans. In the early 1960's, Hasenclever, et al. (27, 28, 29) described 2 serotypes A and B of C. albicans. Since we found that the spectra between strains vary the different spectra may relate to these antigenic groups of C. albicans. Also specific strains of C. albicans appear to change with time in their ability to achieve Y-->M conversion and also in NMR spectra.

Biological applications of NMR are just beginning to be investigated. This study examined the reproducibility of NMR spectra of C. albicans under varied environmental conditions. The study addressed the stability of the organism and the resulting NMR spectra, under prolonged culture passage, animal passage and mixed culture conditions. Experiments that involved different environmental conditions such as media, temperature and growth phase of the organism were

performed. This study establishes base line NMR spectra for further study of the complex events in fungal dimorphism.

MATERIALS AND METHODS

Organisms

Candida albicans strain 394. Strain 394, used in all experiments on morphogenesis, was a gift from R.P. Morrison (University of Oklahoma, Norman, OK). For use in experiments, the yeast was grown in glucose (2%), yeast extract (0.3%), peptone (1%) broth (GYEP) at 37°C under constant aeration by rotation of flasks at 160 rpm (incubator shaker, New Brunswick Scientific, Edison, NJ) and transferred every 48 h. Under these conditions over 99% of the cells were yeast (Y) form. The organism was stored on potato flakes agar (PFA) slants (58) at 4-6°C and transferred every 6 months.

Other strains, species and genera used for NMR analysis. Candida albicans strains 16-149 were obtained from the Clinical Microbiology Laboratories, University Hospital, University of Washington, Seattle, WA. C. albicans strains 158-164 were a gift from Dr. T.G. Mitchell (Duke University, North Carolina). All strains were clinical isolates passed in culture no more than two times before being received. C. albicans strains 172-175 were obtained from the American Type Culture Collection (Rockville, Maryland). C. tropicalis, C. krusei, C. psuedotropicalis, C. guilliermondi and Torulopsis glabrata were from the Montana State University mycologic culture

collection. All organisms were stored on PFA slants. Biochemical confirmation of species was performed on all strains, species and genera by using API strips from Analytab Products, Ayerst Labs (Plainview, N.Y.).

Media

Germination medium (GM-2). GM-2 was prepared from a 10x stock solution (Table 1) by adding 1 part stock to 7 parts sterile deionized water and 2 parts sterile, 0.05M pH 7.4 (at 37°C) tris(hydroxymethyl)aminomethane hydrochloride buffer (pH 7.4 TRIS buffer).

GM-2 with morphogenic autoregulatory substance (MARS). Cell filtrates containing MARS were prepared as previously described from our laboratory (31, 32). Briefly, medium made of 2 parts sterile 0.05M, pH 7.4 (at 37°C) Tris buffer and 8 parts sterile deionized water was inoculated with a 48 h GYEP grown C. albicans 394 (3x washed with sterile 0.15N NaCl) to achieve 2×10^8 Y cells/ml of medium. The cultures were incubated for 15-18 h at 37°C in sterile, glass 1 liter graduated cylinders without shaking. After incubation, suspensions were centrifuged at 1500xg for 2 h and the supernatant fluid was collected and filtered through a 0.45 um porosity membrane filter. The filtrates were lyophilized and

Table 1. Formulation for 10x concentrated GM-2

Ingredients ^a	Amount(mg/l)
folic acid ^b	10
L-arginine HCl	1266
L-methionine	150
L-proline	692
L-histidine	420
glucose	5000
d-biotin	10
(NH ₄) ₂ SO ₄	250
NaH ₂ PO ₄ ·H ₂ O	413
Na ₂ HPO ₄ ·12H ₂ O	717

^a All chemicals are products of Sigma Chemical Company (St. Louis, MO) except glucose and (NH₄)₂SO₄ (J.T. Baker Chemical Company, Philipsburg, NJ)

^b component was heated into solution, the other components were added, and the final solution was sterilized by filtration through 0.45 um porosity membranes (HAWP, Millipore Corp., Bedford, MA)

reconstituted with 1x GM-2 to achieve a 2-4x concentration of MARS.

Synthetic amino acid medium for fungi (SAAMF).

SAAMF at a concentration of 2x was prepared by combining 19.5g of glucose and one packet of SAAM base powder (Table 2) in 500 ml sterile deionized water. The mix was heated to 40-50°C for 1-2 h while stirring, then sterilized by membrane filtration (0.45 um porosity).

SAAMF broth (SAAMFB) was prepared by adding 500 ml of sterile deionized water to the 2x SAAMF, resulting in 1 liter of 1x SAAMFB. SAAMF agar (SAAMFA) was prepared by adding 500 ml of 2x SAAMF to 500 ml 2% agarose (catalog # 27392-444, VWR, San Francisco, CA) in deionized water for a final agarose concentration of 1%.

Germination Assessment

The percent of germination, in experiments involving dimorphism in Candida albicans, was calculated by counting the number of germ tubes in 100 groups of cells. The morphological forms of C. albicans were categorized as germ tubes, pseudotubes, or yeast forms according to previous descriptions (10, 33). A germ tube was defined as a cell with a length at least twice that of the mother cell and with parallel walls throughout

Table 2. Formulation for Synthetic Amino Acid Medium Base

Ingredients ^a	Amount(mg/l)
L-arginine	1050
L-lysine	580
L-histidine	310
L-tyrosine	360
L-tryptophane	100
L-phenylalanine	320
L-cystine	240
L-methionine	150
L-threonine	480
L-leucine	520
L-isoleucine	520
L-valine	460
L-proline	1000
glycine	500
L-glutamic acid	2520
L-asparagine	1000
d-glucose	1000
fumaric acid	1500
sodium pyruvate	1000
ammonium acetate	500
K ₂ HPO ₄ ·3H ₂ O	500

Table 2 (cont'd)

Ingredients	Amount(mg/l)
MOPS ^b	16450
Tris ^c	10450
biotin	0.5
folic acid	5
choline chloride	25
nicotinamide	5
d-calcium pantothenate	25
pyridoxal HCl	5
thiamine HCl	5
riboflavin	5
i-inositol	25
MgCl ₂ anhydrous	95.35
FeCl ₃ .6H ₂ O	2.7
ZnSO ₄ .7H ₂ O	0.8
MnSO ₄ .4H ₂ O	0.36
CaCl ₂ anhydrous	5.57
phenol red	2

^a GIBCO catalog # M00898, all components come premixed from GIBCO (Grand Island, New York)

^b MOPS= 2-(N-morpholino)propane sulfonic acid

^c Tris= 2-amino-2-(hydroxymethyl)-1,3-propanediol

the length. Blastoconidia with no more than 3 attached buds were designated as yeast forms. All other morphologies not fitting into either category were termed pseudotubes.

Cyclic AMP Assay

Preparation of cells. The cells from 100 ml of a 24h C. albicans 394 culture grown in GYEP were washed 3x with sterile 0.15 N NaCl or deionized water. Forty milliliters of GM-2 or GM-2 + MARS in 50 ml Erlenmeyer flasks were inoculated with the washed cell suspension to a final density of 5×10^5 cells/ml of medium. The cultures were incubated at 37°C, 180 rpm in a shaking water bath (Gyrotory Water Bath Shaker, New Brunswick Scientific, Edison, NJ) for between 0.5 and 4 h. [Some difficulty was encountered in optimizing the final protocols. For more information see Appendix II].

cAMP extraction. After incubation, a sample of cells was removed from each culture flask and the percent of germination determined. The cells were collected by membrane filtration (25 mm, 0.45 μ m porosity) and the filter was placed in 2.5 ml of 6% ice-cold trichloroacetic acid (TCA) within 30 sec of removal of the flask from the water bath. The samples were subjected

to 5 cycles of freezing (dry ice: ethanol bath, -70°C) and thawing (water bath: 37°C), filters were removed and the TCA mix was extracted 3x at $23-27^{\circ}\text{C}$ with an equal volume of water-saturated diethyl ether. The remaining ether was evaporated off under a stream of nitrogen gas, and the sample was centrifuged at $1500\times g$ for 15 min. The supernatant fluid was removed, and 2M Tris base was added to achieve a pH of 7. The neutralized supernatant fluid was then lyophilized. The pellets were frozen (-70°C) for use in protein determination.

Protein determination. Each frozen pellet was resuspended in 0.5 ml of 1N NaOH, boiled for 20 min and centrifuged at $1500\times g$ for 15 min. The supernatant fluid was removed and assayed for protein concentration by the method of Lowry (44).

cAMP assay. The lyophilized samples were reconstituted in 0.5 ml of acetate buffer. An ^{125}I cAMP RIA kit [New England Nuclear(NEN), Boston, MA] was used to determine cAMP content of the samples by following the NEN procedure for acetylated samples. (For further enzyme studies see Appendix III.)

Cell Preparation For NMR

Dimorphism studies on *C. albicans* 394. The cells from 500 ml of a 48h *C. albicans* 394 culture grown in GYEP

were washed 3x with sterile 0.15 N NaCl. Eight hundred milliliters of GM-2 or GM-2 + MARS in each of 30 1-liter Erlenmeyer flasks were inoculated at 5×10^5 cells/ml of medium and incubated at 37°C under constant aeration by rotating at 160 rpm. After incubation flasks were placed in an ice-water bath and samples were taken for percent germination assessment. The cells from the 30 1-liter flasks were pelleted by continuous flow centrifugation at $5000 \times g$ in an RC2-B Sorvall centrifuge. The pelleted cells were washed 3x with 0.15 N NaCl and $0.5\text{--}2 \times 10^{10}$ cells were transferred to a 10 mm NMR tube, which was held on ice until NMR analysis.

Strain and genera variation. From cultures maintained at $4\text{--}6^\circ\text{C}$ on PFA slants, 2 ml of SAAMFB in 5 ml tubes were inoculated and incubated at 37°C overnight under constant aeration by rotating at 160 rpm. One hundred and fifty milliliters of SAAMFB in 250 ml Erlenmeyer flasks were inoculated with 2 drops of the overnight culture and incubated at 37°C , 160 rpm for 24h. For growth curve experiments the 150 ml of SAAMFB was inoculated to a total cell density of 10^8 cells from 24h cultures grown in 10 ml of SAAMFB. Cells obtained for 28°C temperature experiments were grown in a shaking water bath at 160 rpm. Cells were collected, washed 3x

with 0.15 N NaCl and $0.5-2 \times 10^{10}$ cells were packed into a 10 mm (OD) NMR tube, on ice.

NMR of mixtures of *C. albicans*. One hundred and fifty milliliters of SAAMFB in 250 ml Erlenmeyer flasks were inoculated with *C. albicans* 16 and *C. albicans* 113, 109 or 122 and incubated at 37°C, 160 rpm for 24h. Strain 16 was used because it does not agglutinate with an agglutinating monoclonal antibody (H-9, # 35 antibody isolated in Dr. Cutler's lab by Diane Brawner). The other strains (113, 109, and 122) used always gave a 3 or 4+ agglutination with the antibody. After incubation, the mixed cultures were plated onto SAAMFA and incubated at 37°C for 24 h. Isolated colonies were tested with the antibody and specific colonies were chosen on the basis of agglutination. Cells from these colonies were used to inoculate SAAMFB. The cultures were grown for NMR analysis following the strain and genera procedures.

NMR of *C. albicans* passed through mice. The cells from 10 ml of a 24 h SAAMFB culture of different *C. albicans* strains were washed 3x with sterile 0.15 N NaCl, and suspensions of 1×10^7 cells/ml were prepared. BALB/cByJ mice, obtained from Jackson Laboratories (Bar Harbor, ME), were injected intravenously with 0.1 ml of the washed cell suspensions. The mice were sacrificed 3 days after injection. The kidneys of the mice were

removed, homogenized and plated on SAAMFA following the procedure in our lab (15). The plates were incubated at 37°C for 24 h and cells from isolated colonies were used to inoculate 150 ml SAAMFB in 250 ml Erlenmeyer flasks for incubation following strain and genera procedure.

NMR Parameters

^{13}C NMR spectra of the cells were recorded at 2°C by means of a Bruker WM-250 spectrometer working at 62.83 MHz, interfaced to an Aspec-2000 computer, under conditions of broad band decoupling. The field/frequency ratio of the spectrometer was locked onto a coaxial D_2O sample and referenced with an internal tetramethylsilane (TMS) capillary. For the dimorphism studies, each sample was recorded as an 8K data file of 5000 accumulated free induction decays (FIDS) obtained by 55° pulses with an acquisition time of 0.2 sec, relaxation delay of 0.2 sec and sweep width of 20000 Hz. Strain and genera variation experiments were stored as 8K data files obtained by 600 scans of 85° pulses with an acquisition time of 0.54 sec, relaxation delay of 3 sec and sweep width of 15151.51 Hz. The 8k FIDS were truncated to 4k, Fourier transformed and then the power spectra were obtained and used in evaluation.

RESULTS

Cyclic AMP and Dimorphism

To determine if cyclic AMP (cAMP) plays an important role in morphogenesis using the system of MARS suppression of germination (Y-->M conversion), intracellular levels of cAMP were evaluated. Candida albicans strain 394 (5×10^5 cells/ml) in duplicate flasks of GM-2 or GM-2 + MARS were incubated at 37°C for 0.5, 1 and 3 h time periods. After incubation, the cells were collected, lysed, extracted and centrifuged. The supernatant fluid was analyzed with an ^{125}I -cAMP radioimmune assay kit (New England Nuclear) to determine intracellular levels of cAMP. There was no significant difference in cAMP levels (Figure 1) between germinating (GM-2) and germination-suppressed (MARS-treated) cells noted at the early time periods (0, 0.5 and 1 h). Multiple repetitions of this experiment, as well as experiments looking at other intermediate time periods, all revealed no increase in cAMP. Only when the Y-->M conversion was morphologically complete at 3 h did the cAMP in the M form cells increase.

NMR and Dimorphism

To determine if different morphological forms of Candida albicans have different NMR spectra, 5×10^5

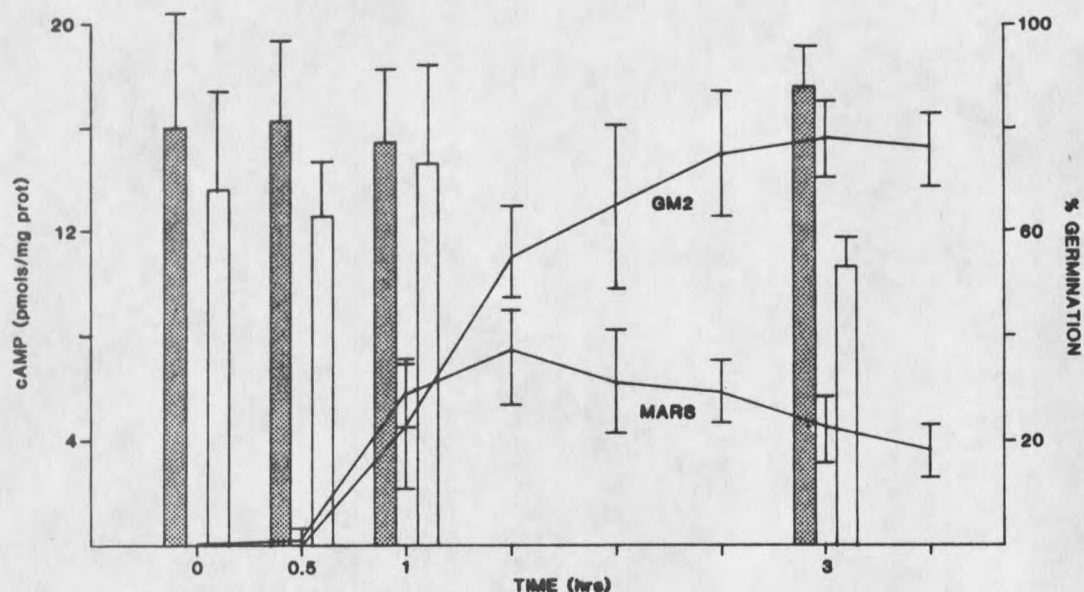


Figure 1. Germination and intracellular cyclic AMP levels of *Candida albicans*. The bars indicate levels of cAMP assayed at 4 different time periods. Shaded bars are cAMP levels of cells under germinating conditions (GM-2). Open bars are levels under conditions of suppressed germination (GM-2 + MARS). Curves represent percent germination measured every 0.5 h of the experiment.

cells/ml in flasks of GM-2 or GM-2 + MARS were incubated at 37°C for 3 h. The cells were collected, washed and packed into a 10 mm (OD) NMR tube. The spectra obtained (Figure 2) were reproducible over multiple repetitions when percent germination and morphologies between runs were similar, and the temperature in the NMR sample was 2°C. The Y control spectrum was obtained on Y cells that were not released into GM-2 or MARS + GM-2. Since the Y control cells were grown in GYEP but not released into GM-2, these cells served as a control of the media difference from maintenance in GYEP to experimentation in GM-2 by noting peak differences in the spectra. The control cells also showed any changes in the strain that had occurred over continuous passage in GYEP. The base line spectra obtained serve as a source of comparison between runs and as a control see that the spectrometer is running properly.

For ease of discussion of figures, all spectra were divided into 3 sections. Section I (10-35 ppm) was referred to as the aliphatic region. This region contains most of the peaks for aliphatic carbons, for example methyl side chains on amino acids and methyl and methylene carbons on fatty acids (1, 42, 48). The region of peaks due to the hydroxyl, ether, sulfhydryl and amine

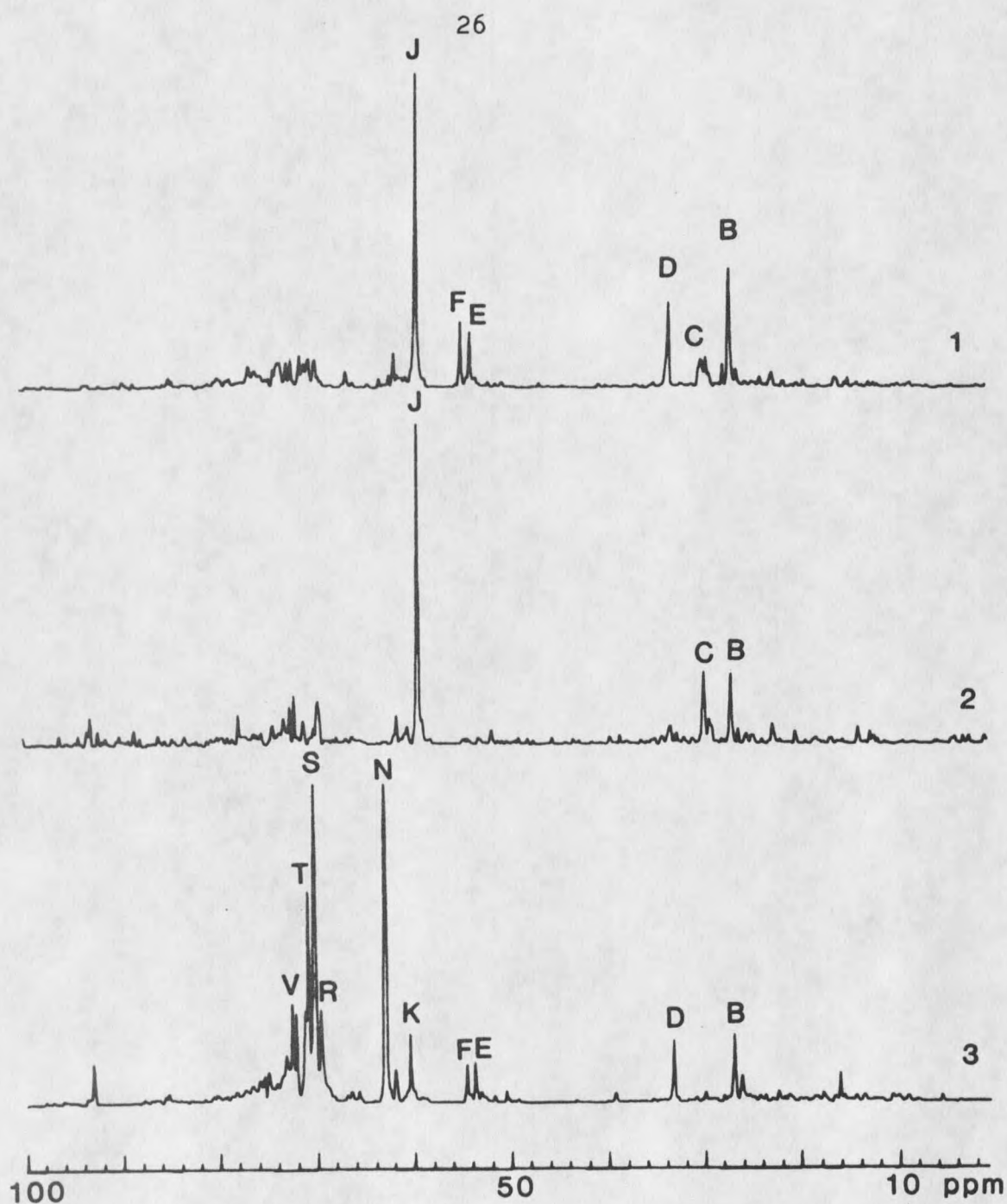


Figure 2. ^{13}C NMR spectra of 3 h incubated Candida albicans cells. 1) suppressed germination (MARS), 2) germinating (GM-2) and 3) control or 0 h cells. Letters designate specific peaks, the chemical shifts (ppm) of which were referenced to TMS.

linkages to carbon was referred to as the carbohydrate region (1, 42, 48). The carbohydrate region was divided into section II (50-65 ppm) and III (65-85 ppm). Within all of these sections individual peaks were labelled with letters. The letter designations on all figures are the same and therefore the same letter in any 2 spectra, on any 2 figures refer to the same peak.

The signal/noise ratio was also used to compare spectra. Noise is the random background of peaks due to both the instrument and the environment. Peaks that are 2 times the noise level are considered real. Since noise is a constant, the signal/noise ratio of any specific environmental conditions and cells was consistent (67).

There were similarities and differences between the MARS-treated cells (Y), M (GM-2) and Y control spectra. Section III (65-85 ppm) and peaks J (59.5 ppm), K (60.6 ppm) and N (63.1 ppm) in the carbohydrate region are examples of differences apparently due to media. There was a reduction in section III and peaks K and N but an increase in peak J in the GM-2 and GM-2 + MARS released cells as compared with the Y control cells grown in GYEP. Peak B (27 ppm) in the aliphatic region is an example of a peak that is constant in all 3 spectra. Whereas peaks C (30.7 ppm), D (33.2 ppm), E (54.3 ppm) and F (54.8 ppm) depend on the morphology of C. albicans. A reduction in

peak C and an increase in peaks D, E and F were observed in Y spectra under control conditions and MARS-treated cells. The reverse was true for M form cells which gave reduced peaks D, E and F and an increase in peak C.

The second NMR experiment was to examine early time periods during morphogenesis in an attempt to detect compounds which correlate with initiation of morphogenesis. The NMR spectra of cells grown in GM-2 or GM-2 + MARS for 10 min are shown in Figure 3. Although after the 10 min incubation period both GM-2 and GM-2 + MARS appear the same morphologically, there were differences in the NMR spectra. Peaks B (27 ppm) and D (33.2 ppm) in the aliphatic region and peak N (63.1 ppm) in the carbohydrate region were reduced under germinating conditions in comparison with the Y cells in either MARS-treated or control conditions. Changes were also noted in the relative peak heights of peaks T (70.8 ppm), Y (73.2 ppm) and Z (76.1 ppm) under germinating conditions by 10 min of incubation.

NMR Spectra of Other Yeasts

NMR spectra of 4 other species of Candida (C. krusei, C. tropicalis, C. pseudotropicalis and C. guilliermondi) and one member of the genus Torulopsis

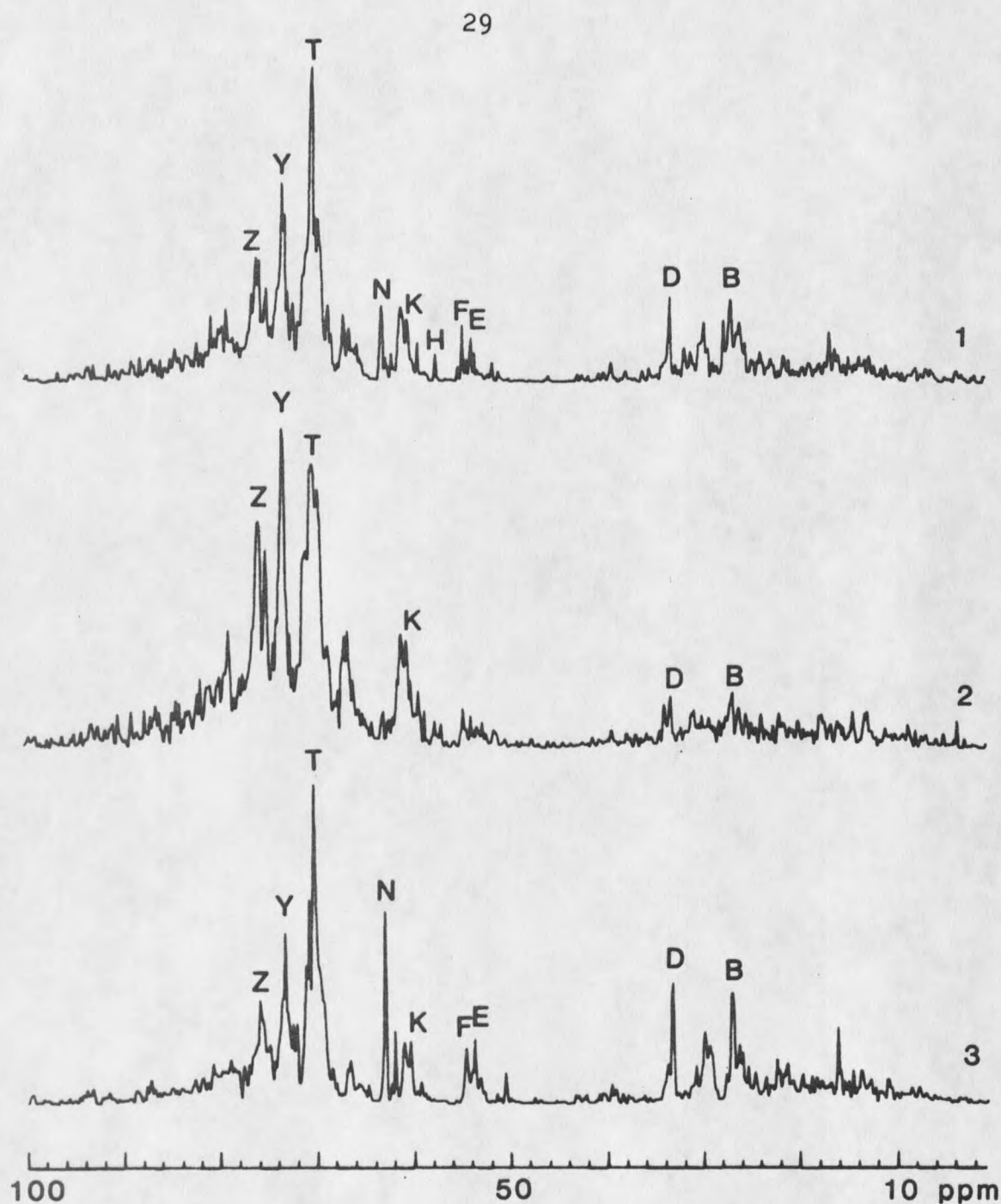


Figure 3. ^{13}C NMR spectra of 10 min incubated *C.albicans* cells. 1) suppressed germination (MARS), 2) germinating (GM-2) and 3) control or 0 h cells. Letters designate specific peaks, the chemical shifts (ppm) of which were referenced to TMS.

(T. glabrata) were compared with C. albicans NMR spectra. All cells grown under the standard conditions of 24 h incubation in SAAMF at 37°C showed significant differences in NMR spectra. C. tropicalis and C. krusei gave consistently better signal/noise ratios than C. guillermondii (Figure 4). C. guillermondii has peaks of greater intensity in the aliphatic region I (10-35 ppm) than the other 2 species shown. There also are different ratios of peaks in the carbohydrate region II (50-65 ppm) and III (65-85 ppm) in the 3 species. For example, in C. tropicalis the peaks in section III are greater in intensity than section II, whereas the reverse is true for the other 2 species. Figure 5 is a comparison of the spectra of C. albicans, T. glabrata and C. pseudotropicalis. The aliphatic region I is more pronounced in these species as compared with the 3 species in Figure 4. The relative peak heights of peaks in the carbohydrate regions II and III are similar in T. glabrata and C. albicans, however the individual peaks vary notably.

Strain Variation NMR

To determine if all strains of C. albicans have the same NMR spectra and if identification and fingerprinting of the strains was possible, NMR spectra from 25 strains

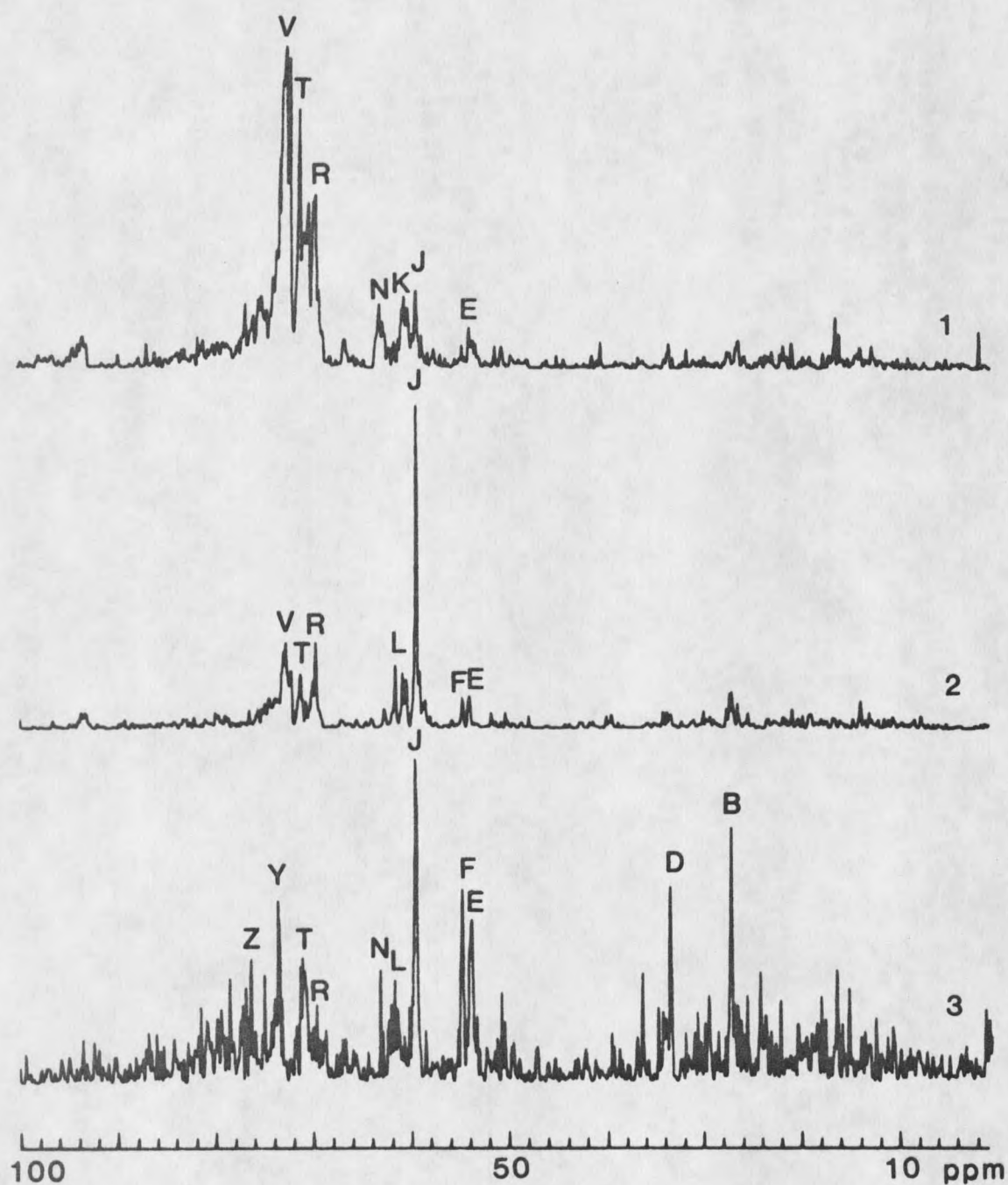


Figure 4. ^{13}C NMR spectra of different species of *Candida*. 1) *C. tropicalis*, 2) *C. krusei* and 3) *C. guilliermondii*. Letters designate specific peaks, the chemical shifts (ppm) of which were referenced to TMS.

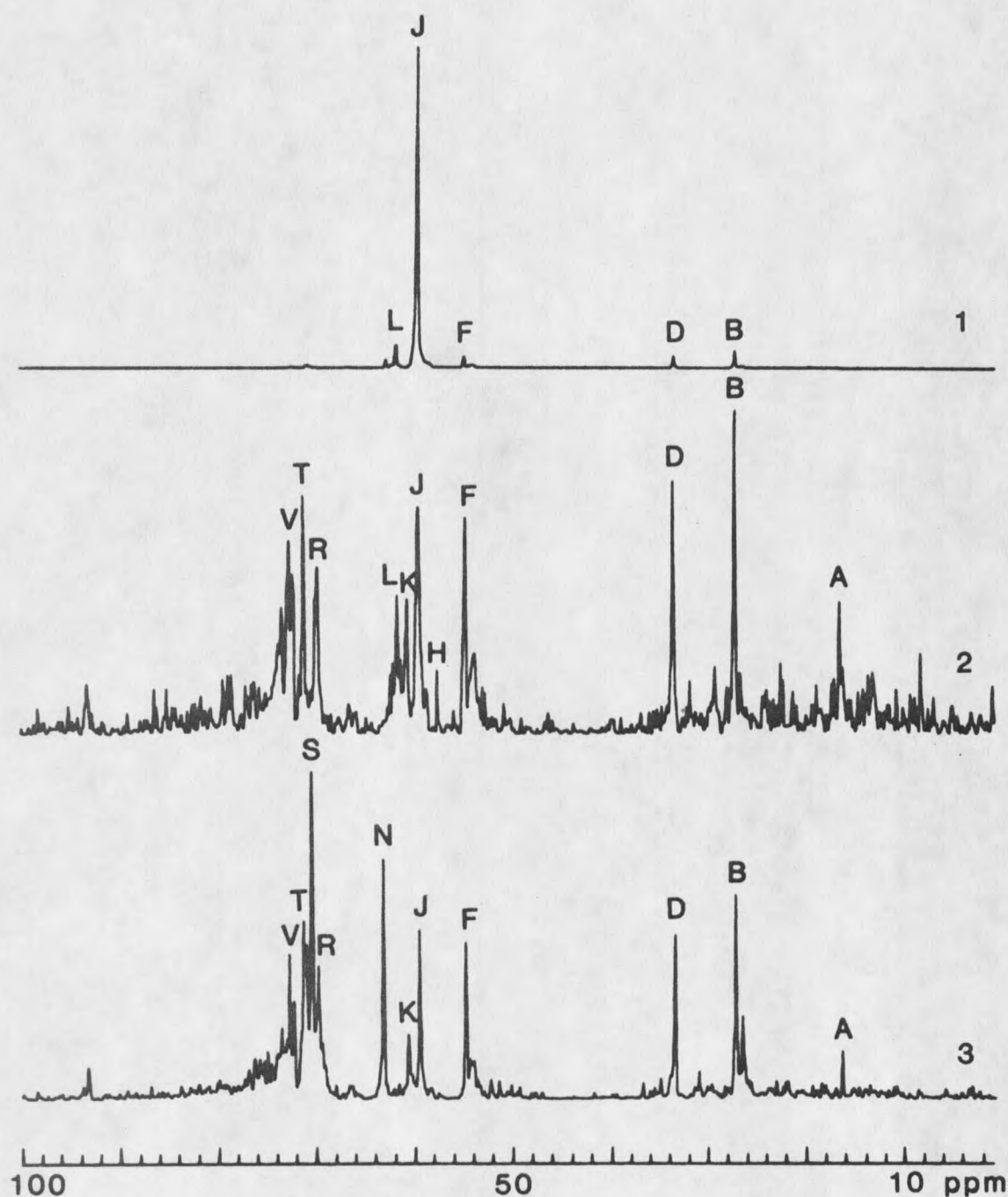


Figure 5. ^{13}C NMR spectra of different species and genera. 1) *Candida pseudotropicalis*, 2) *Torulopsis glabrata* and 3) *C. albicans* (118). Letters designate specific peaks, the chemical shifts (ppm) of which were referenced to TMS.

of C. albicans were analyzed. All strains were grown under the standard conditions of a 24 h incubation in SAAMF at 37°C. No identification of individual strains was possible due to the range of intensities observed in multiple repetitions of the same strain. However, 3 major spectral patterns (designated type 1, 2 and 3, Figure 6) emerged from this analysis (Table 3). Nineteen strains of C. albicans produced the type 1 spectrum which was defined by the increased complexity in carbohydrate region III (65-85 ppm). In addition other peak differences were noted among the 3 spectral types in section II (50-65 ppm) of the carbohydrate region. C. albicans strain 118 was used as the typical type 1 organism. Type 2 was defined as spectra with few or no peaks in the aliphatic region I (10-35 ppm), 3 notable peaks in the carbohydrate regions II and III and generally few peaks in the spectra overall. The signal/noise ratio in type 2 spectra was consistently better than type 1 or 3. C. albicans strain 122 was used as the typical type 2 organism in further studies. Five of the C. albicans strains fell into the type 2. One strain, 117, had a different enough spectrum to be put into a third group of spectra. Type 3 spectra are characterized by no notable peaks in the carbohydrate region III, but significant peaks in section II.

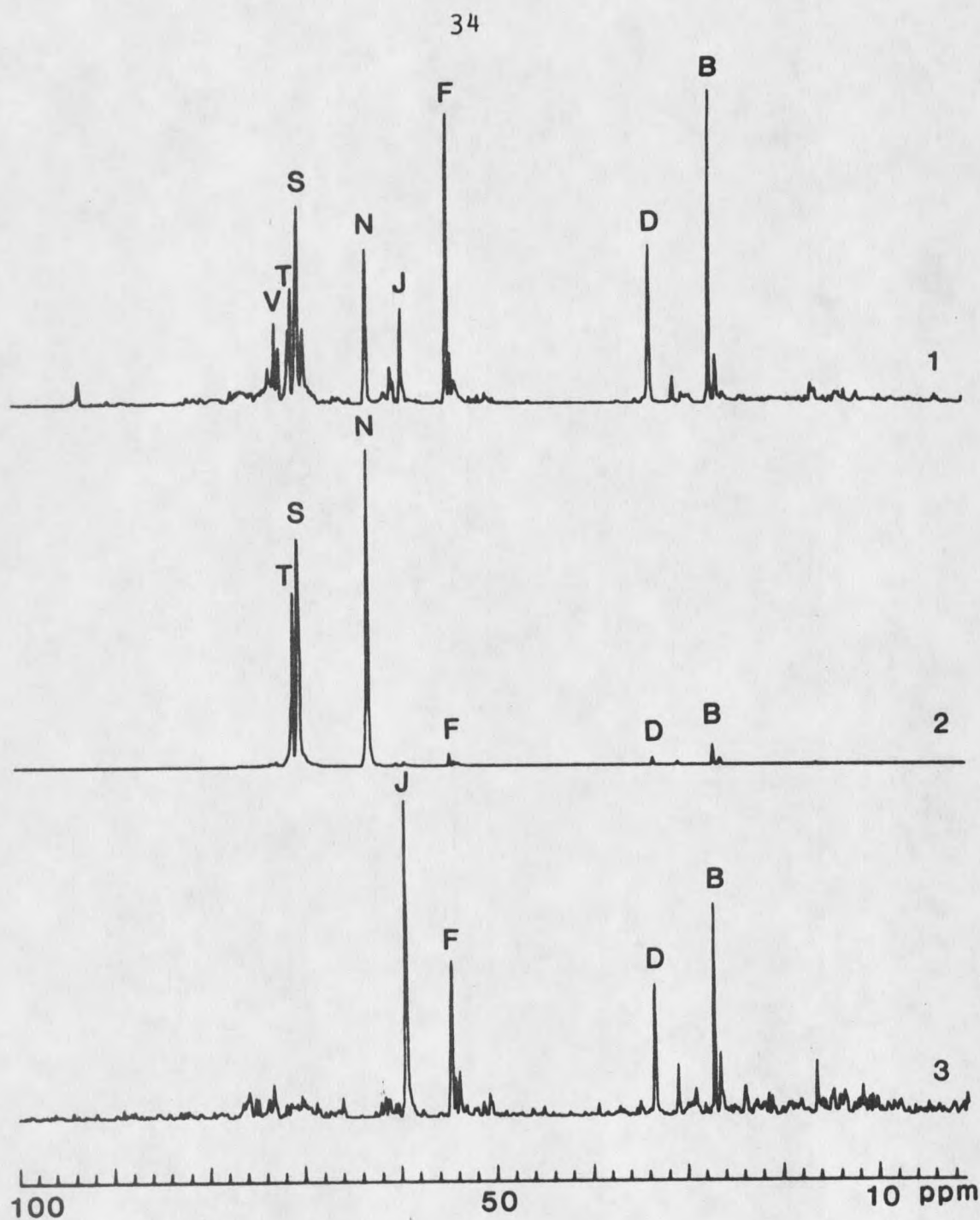


Figure 6. ^{13}C NMR spectra of the 3 types of *C. albicans* spectra. 1) type 1 (*C. albicans* 118), 2) type 2 (*C. albicans* 122) and 3) type 3 (*C. albicans* 117). Letters designate peaks, the chemical shifts (ppm) of which were referenced to TMS.

Table 3. C. albicans strains and spectral types

NMR spectral types	<u>C. albicans</u> strains ^a
1	16 (5), 104 (9), 105 (3), 112 (1), 115 (1), 118 (33), 129 (2), 130 (1), 131 (1), 138 (2), 149 (2), 158 (2), 159 (2), 160 (2), 161 (2), 163 (2), 164 (2), 9938 (1)
2	109 (6), 113 (3), 122 (27), 135 (8), 147 (6)
3	117 (4), 172 (3), 173 (2)
4	174 (2), 175 (2)

a = the number in the parentheses refers to the total
number of spectra obtained from the given strain

The grouping of C. albicans strains on the basis of NMR spectral patterns led to questions as to whether this grouping related to the antigenic typing of C.albicans serotypes A and B. Four known A and B serotyped organisms were obtained from ATCC and grown under standard conditions for NMR analysis. The type B organisms were grouped with type 3, since the spectra were similar. The type A's had some similarities with the other types, but did not fall into any one particular type. Therefore a fourth type was designated. However there are too few organisms and not enough spectra to establish a base line spectra.

Environmental Effects on Spectra

Various strains of C. albicans were tested to see if similar spectra would be obtained under different environmental conditions. The first environmental factor looked at was the growth phase of the organism. The cells for the growth curve experiments were grown under the standard conditions of SAAMF at 37°C, but incubated for different lengths of time. Since the cells were grown in SAAMF before transfer to SAAMF for this experiment, there were no media effects in the spectra. Figure 7 shows spectra of 3 time periods (4, 7 and 20 h) in the growth of C. albicans 122 (type 2). The signal/noise ratio is

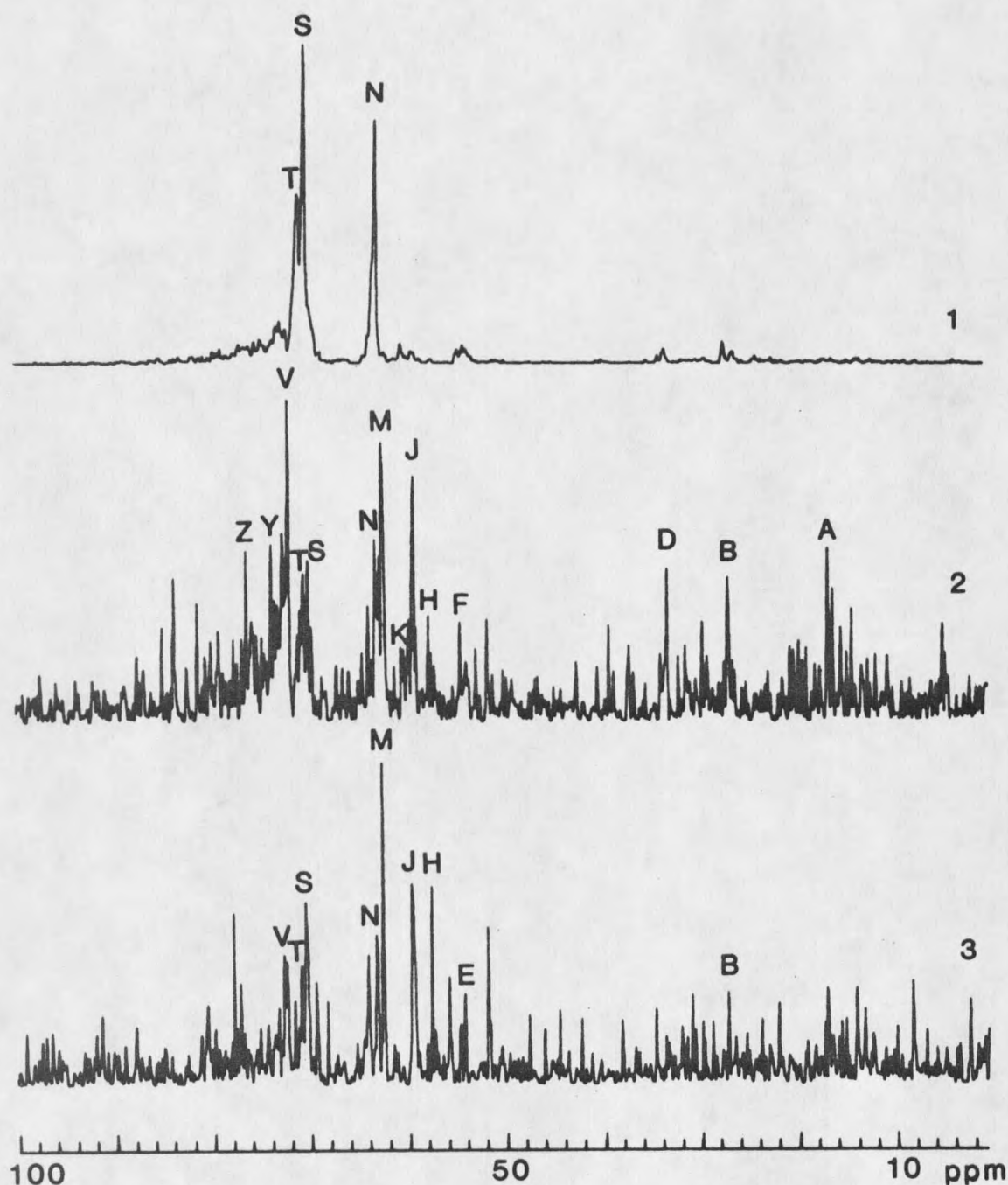


Figure 7. ^{13}C NMR spectra of 3 different time periods in the incubation of *C. albicans* 122 (type 2). 1) 20 h, 2) 7 h and 3) 4 h incubations. Letters designate specific peaks, the chemical shifts (ppm) of which were referenced to TMS.

significantly better in the later time periods (20 h). It is possible to note the increase in peaks N (63.1 ppm) and S (70.4 ppm) in the carbohydrate region that were noted at the later time periods along with a decrease in most other peaks. In this strain of C. albicans the spectrum achieved by 20 h doesn't change through at least 48 h.

Figure 8 shows spectra of C. albicans 118 (type 1) at 3 different time periods (7, 20 and 24 h) in the growth curve. Again the signal/noise ratio was better in the later time periods (20 and 24 h). The aliphatic region shows notable changes with an increase in peaks B (27 ppm) and D (33.2 ppm) with time. Within the carbohydrate region II (50-65 ppm) there was a constant peak K (60.6 ppm), peaks that increased with time like F (54.8 ppm) and N (63.1 ppm), and peaks that decreased with time like peak H (57.6 ppm). Peak J (59.5 ppm) first decreased and then later increased with time. Peaks S (70.4 ppm) and T (70.8 ppm) decreased in peak height at 20 h, but then increased at 24 h, whereas peak V (72.2 ppm) decreased with time. Unlike the growth curve NMR analysis for 122 however, the NMR pattern of 118 continued to change as a function of cell incubation time on out to the test limit of 48 h.

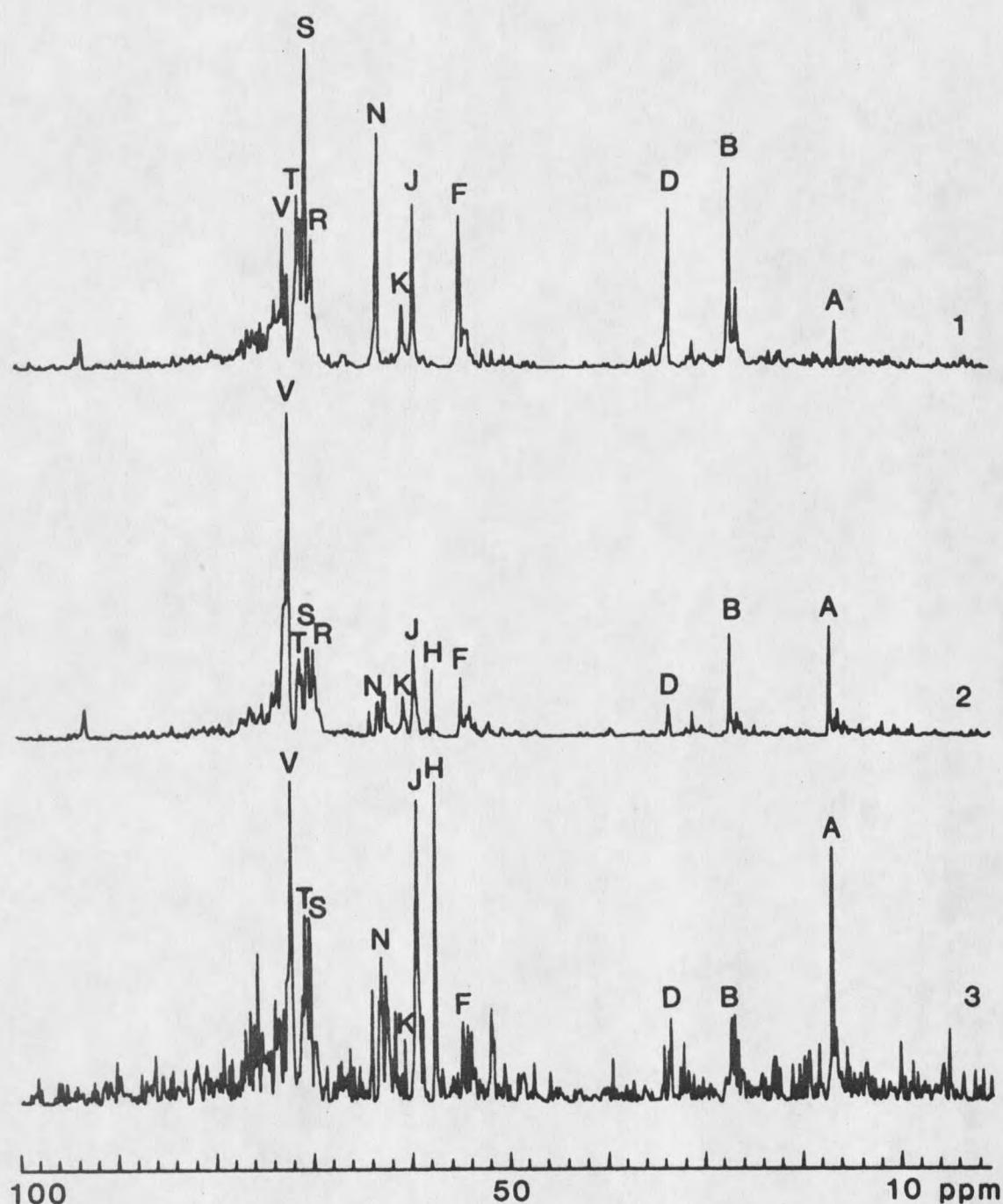


Figure 8. ^{13}C NMR spectra of 3 different time periods in the growth of *C. albicans* 118 (type 1). 1) 24 h, 2) 20 h and 3) 7 h incubations. Letters designate specific peaks, the chemical shifts (ppm) of which were referenced to TMS.

Next the similarity of spectra from the same C. albicans strain in different media with all other conditions the same was studied. The 3 media used were SAAMF (see Table 2), GYEP (glucose, yeast extract and bacto-peptone) and Sabouraud dextrose broth (neo-peptone and glucose). Figure 9 shows spectra of C. albicans 118 (type 1) in these 3 media. There were some similarities between the 3 media in the aliphatic region. For example, peaks B (27 ppm) and D (33.2 ppm) had relatively the same peak heights in all 3 media. The carbohydrate region differed however. Peaks F (54.8 ppm), J (59.5 ppm) and K (60.6 ppm) varied in relative heights to each other in the different media and the peaks varied in height between spectra. Section III (65-85 ppm) in the carbohydrate region was the most different in both the set of peaks and relative intensities. The signal/noise ratio was significantly better for organisms grown in GYEP and SAAMF than in Sabouraud broth.

The next environmental factor tested was different incubation temperature. Two different strains of C. albicans (a type 1 and a type 2) were incubated in SAAMF, for 24 h at 28° and at 37°C. NMR spectra obtained from analysis of C. albicans 122 (type 2) was essentially the same for cells incubated at 28° or 37°C, therefore only the 37°C incubation spectrum was shown (Figure 10).

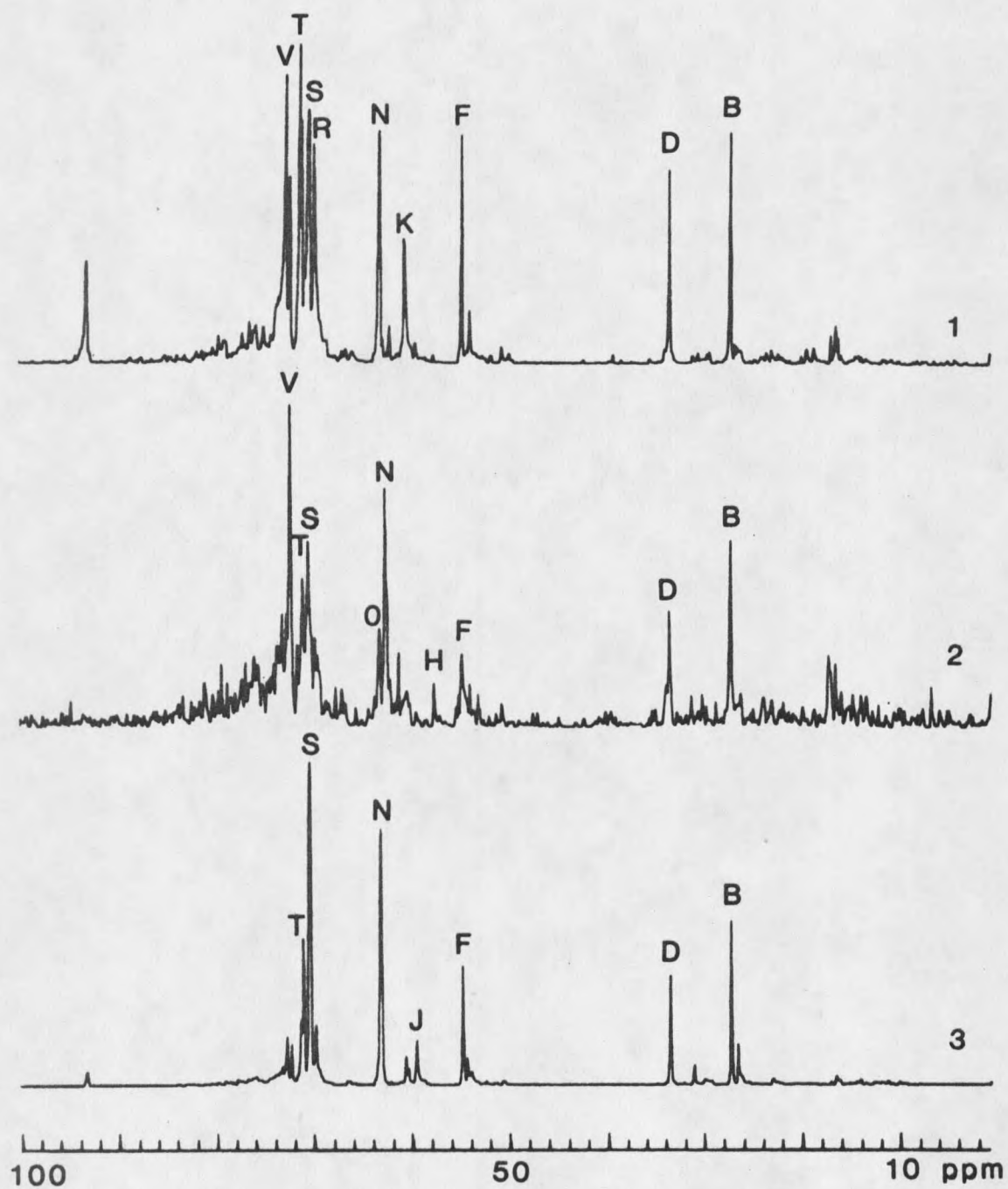


Figure 9. ^{13}C NMR spectra of *C. albicans* 118 in 3 different media. Cells incubated in 1) GYEP, 2) Sabourauds Dextrose and 3) SAAMF. Letters designate specific peaks, the chemical shifts (ppm) of which were referenced to TMS.

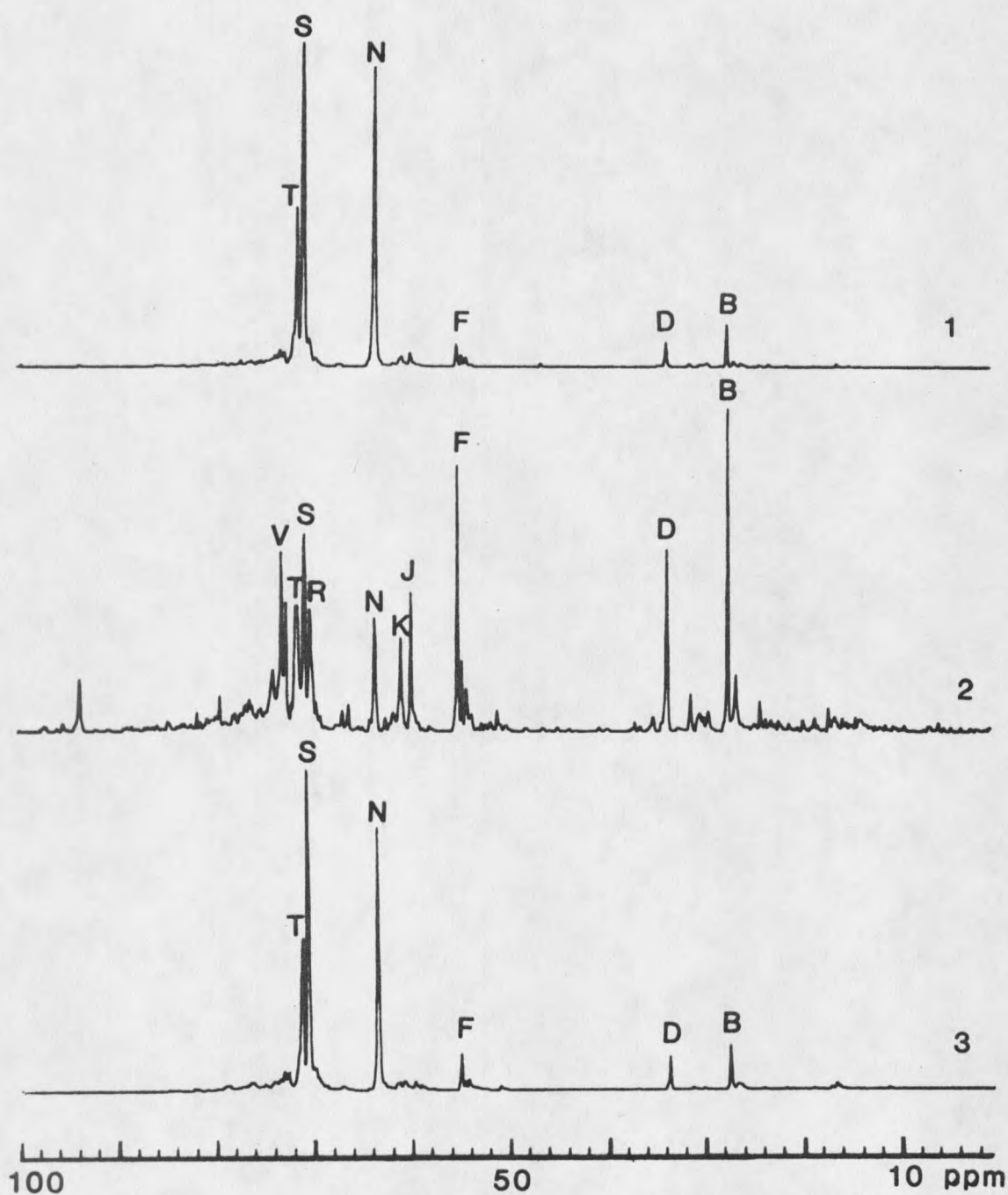


Figure 10. ^{13}C NMR spectra of *C. albicans* incubated at 2 different temperatures. 1) *C. albicans* 122, 37°C , 2) 118, 37°C and 3) 118, 28°C . Letters designate specific peaks, the chemical shifts (ppm) of which were referenced to TMS.

C. albicans 118 (type 1) was very different for the 2 incubation temperatures. The aliphatic region I (10-35 ppm) was markedly decreased in the 28°C spectrum over the standard 37°C. The carbohydrate regions II (50-65 ppm) and III (65-85 ppm) showed a reduction in the number of peaks as well as differences in peak heights in the 28°C spectra. The resulting 28°C 118 (type 1) spectrum looked very similar to a standard 37°C 122 (type 2) spectrum.

NMR of Other Effects on the Cells

Several different experiments were done to determine if spectra were stable when C. albicans cells were incubated under standard conditions but with other variables. One experiment involved the incubation of 2 different strains of C. albicans in the same flask of SAAMF for 24 h, at 37°C. Strains were separated by plating and isolating colonies on SAAMF. Isolated colonies were then tested with a monoclonal antibody to identify the 2 strains. Then NMR spectra of both were obtained. C. albicans strains were also injected into mice and the spectra before and after mouse passage were compared. Another experiment involved the continual passage of strains of C. albicans in SAAMFB over a period of months. The NMR spectra of the cells was taken periodically and compared for changes.

All 3 experiments showed there were changes in the spectra (figure 11). There was little effect in the aliphatic region of any of the spectra, as was seen in peaks B (27 ppm) and D (33.2 ppm) which were similar in all 3 spectra. However there was a major difference in peak A (16.7 ppm) which was increased after mouse passage. The mouse passage spectra showed the most notable changes of all the spectra. For example, peaks F (54.8 ppm) and J (59.5 ppm) had similar peak ratios in the standard and continuous passage, but the peaks were reversed in the mouse spectra. However, there were similarities in the carbohydrate region, evidenced by the ratio of peaks R (69.7 ppm) to K (60.6 ppm). These peaks were similar in all 3 spectra. Section III (65-85 ppm), had similar peaks in all 3 spectra, although peak heights varied especially in the continuous passage spectrum. The continuous passage spectrum had a lower signal/noise ratio than the standard spectrum. The mixed strain NMR, although not shown, had a spectrum similar to the continuous passage spectrum.

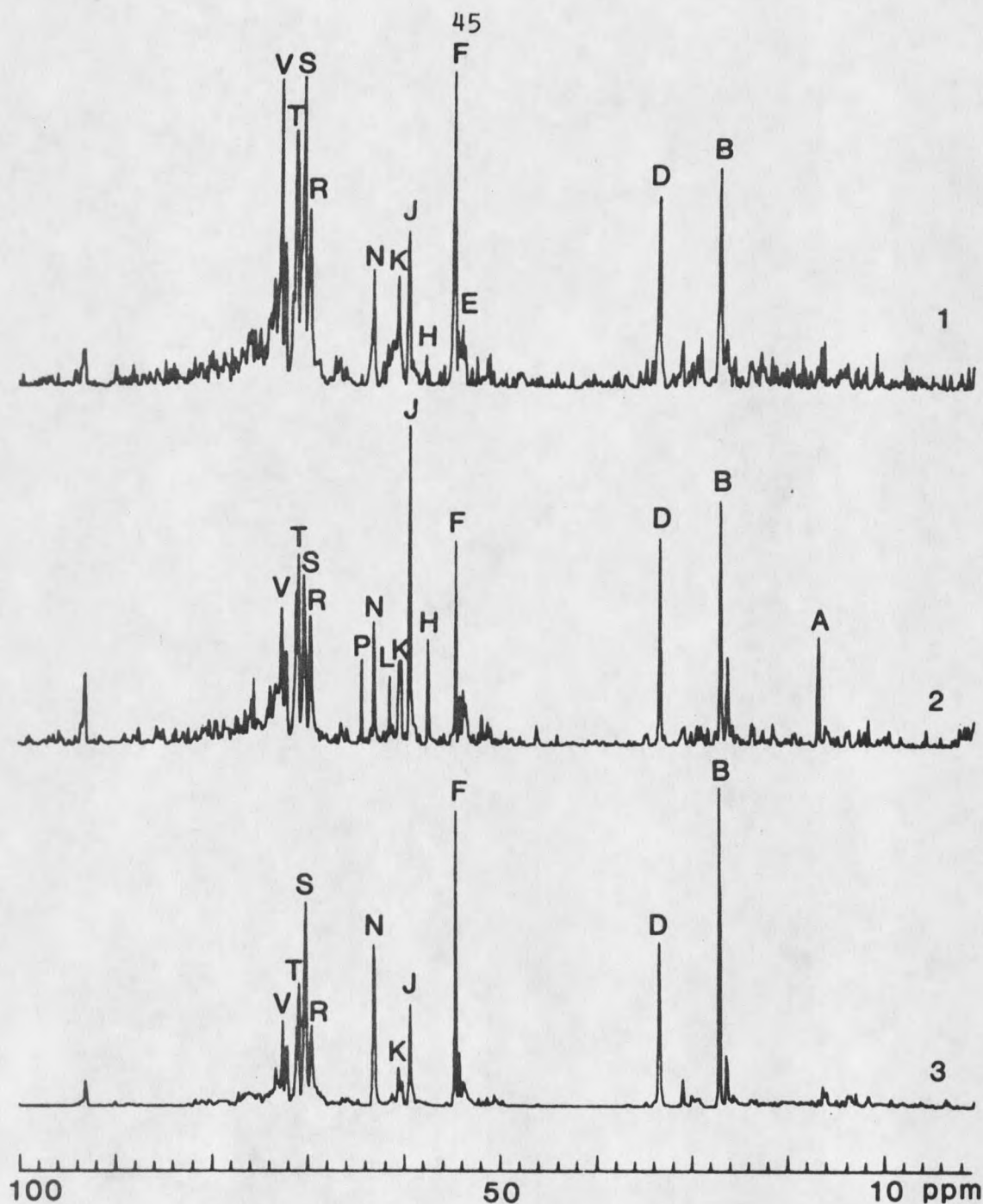


Figure 11. ^{13}C NMR spectra of *C. albicans* 118 incubated under other environmental conditions. 1) continuous passage cells, 2) mouse passage cells and 3) a standard spectrum. Letters designate specific peaks, the chemical shifts (ppm) of which were referenced to TMS.

DISCUSSION

Dimorphism in Candida albicans is a complex problem. Specific factors that regulate the morphogenic process have been studied (24, 35, 50, 56, 63, 75, 76). However, investigators have used many different strains of organisms, media, temperatures for growth and methods to achieve yeast (Y)--> mycelial (M) conversion. Correlation and comparison of all of the data have been difficult because of these many different protocols.

One of the main differences in protocols of various investigators is the method used to induce a Y-->M conversion. The most common induction is by temperature shift, which involves changing the incubation temperature from approximately 28° at which Y are maintained, to 37°C to achieve the Y-->M conversion. The temperature shift approach was used by both Niimi, et al. (53) and Chattaway, et al. (14) to look at cyclic AMP levels in the Y and M forms of C. albicans. However this temperature shift probably does not reflect the in vivo situation of a normal flora organism causing an opportunistic disease because both the saprobic (Y) and parasitic (Y and M) forms of C. albicans grow at 37°C. Therefore cAMP in C. albicans was reevaluated using MARS to suppress the Y-->M conversion rather than a 25°C temperature.

In using MARS as a suppressor of germination both Y and M cells were grown at the same temperature of 37°C. A defined minimal medium (GM-2) was used to grow the Y and M forms. The addition of MARS which is produced by Y form cells to maintain Y cells is the only difference in Y and M conditions. The MARS system is therefore a well defined system, using a nutritional shift rather than temperature shift. The MARS system is important in order to understand how the morphogenic process occurs in humans under conditions which favor tissue invasion, namely 37°C and nutritional shifts.

Data obtained from using MARS as a suppressor of germination indicated that there was no significant difference in cAMP levels at the early time periods studied. If cAMP was involved in the initiation of the morphogenic conversion, increased levels of cAMP would be expected before any morphologic signs, however this was not found to be true. Niimi, et al. (53) and Chattaway, et al. (14) reported an increase in cAMP at approximately one hour into the Y-->M conversion. Our data support the fact that an increase in cAMP was seen in the M form cells at 3 h or at a time when phenotypic expression of morphogenesis is noted. However this is not evidence that cAMP is important as an initiation factor.

It is also important to note that the cAMP studies as well as many other typical biochemical approaches are in vitro assays that entail breaking cell membranes and therefore use invasive methods. NMR is an alternative method that is not as sensitive, but has the advantage of being able to be used to study live cells without disrupting cellular integrity.

Cassone, et al. (9) was the first group of researchers to look at dimorphism in C. albicans using NMR spectroscopy. These workers used ^{31}P NMR spectroscopy in an attempt to study the complex problem of dimorphism by looking for differences in the Y and M forms. However, Cassone et al. (9) used two different media and two different temperatures to grow the Y and M form of C. albicans. Since, as we have found, NMR spectroscopy is very sensitive to differences in the nutritional and physical environment of the cells to be studied, experiments addressing the effect of the different temperatures and media should have been done. There must be control spectra of temperature and media effects in order to tell which compounds in a spectrum are due to dimorphism only.

The MARS system contains a control for the changing medium from Y maintenance to Y-->M inducing medium. Yeast cells to be used in NMR experiments were maintained at

37°C in GYEP. These cells were washed and introduced into GM-2 or GM-2 + MARS for a given time and then NMR spectra obtained. Spectra from cells in GM-2 (germinating), GM-2 + MARS (suppression of germination) and also spectra for Y cells from the inoculum were obtained. The Y control spectra when compared with the Y MARS spectra should indicate compounds that appear because of nutritional shift from the GYEP medium to GM-2. Therefore compounds in the GM-2 cells but not in MARS or the Y control are compounds that are important for dimorphism.

^{13}C studies of 3 h cells using this system indicated that compounds were specific for the M and Y forms as is evidenced by the differing peaks between spectra in Figure 2. Peaks D (33.2 ppm), E (54.3 ppm) and F (54.8 ppm) are evident in the Y spectra, whereas peak C (30.7 ppm) increased in the M form cells. NMR spectra of Y and M cells from the early time periods (10 and 20 min) have indicated that there are specific compounds that initiate morphogenesis (Figure 3). The change in ratio of peaks T (70.8 ppm), Y (73.2 ppm) and Z (76.1 ppm) in section III (65-85 ppm) of Y and M spectra indicated there were different carbon intermediates formed, but identification of these has not been done.

Although the above experiments were interesting their interpretation was difficult because of the following problems associated with Y-->M conversion studies in general and because of the lack of solid base line data considering the stability of NMR patterns. When a Y cell undergoes conversion to the M form there are many forms in which the cells can appear. The morphological forms of C. albicans were categorized as germ tubes, pseudotubes, or yeast forms (10, 33). The group of cells called pseudotubes can vary greatly in morphology since they are all of the morphologies not fitting into either of the other two categories. Converting 100 Y cells into 100 true germ tubes is usually not possible even under ideal conditions. The percentage of germ tubes can also vary from experiment to experiment and seems to be governed by as yet unidentified factors. The NMR detects these different forms of varying morphologies in terms of their varying biochemistries. Until morphogenesis is more standardized in the lab, NMR may not be the best technique to study dimorphism.

To effectively use NMR in studies on dimorphism it is also important to understand how to achieve reproducible spectra. That is, for a given strain under a set of conditions, how reproducible were the NMR patterns

taken at various times? During the course of this research a strain of C. albicans under intensive study had unexplainably stopped germinating and thus another strain of C. albicans was substituted to allow continuation of the experiments. It was noted, however that the NMR spectra of the 2 different strains were similar but not identical. Examination of earlier data showed that the Y form control cells of the strain used in the initial studies seemed to have a changing spectrum with time.

While using NMR spectroscopy to study Candida morphogenesis, problems like those mentioned above were encountered because of apparent instability of spectra. This led to several questions about factors that will ultimately effect the NMR spectra. First, we wanted to see if the NMR patterns seen with C. albicans were unique or if all Candida sp. would have similar spectra. Second, if the spectrum of C. albicans was unique when compared to other species, how do individual strains compare? It was noted that the NMR spectra of several different strains of C. albicans were different. Perhaps identification of different strains would be possible through NMR. Third, what environmental factors that affect the growth of C. albicans will also affect the

spectra? Growth phase, temperature of incubation and media differences were all looked at. The last questions dealt with whether continuous media passage of cells, mixed culture or mouse passage of cells would effect the NMR patterns.

In setting out to answer the questions posed and study the factors involved, a standard system was devised to obtain the cells for NMR analysis. A defined medium (SAAMF), and a 24 h incubation at 37°C were chosen as the parameters for the studies. Maintenance of the stock cultures was on slants that were stored at 4-6°C, rather than continuously transferring the cells.

To answer the first question of how do different species of Candida spectra compare, several species of Candida (C. krusei, C. pseudotropicalis, C. tropicalis and C. guillermondii) and Torulopsis glabrata were looked at with NMR spectroscopy. The spectra were reproducible for each species and differed between species (Figure 4,5). In comparison to C. albicans, the aliphatic region (10-35 ppm) of C. tropicalis, C. krusei and C. pseudotropicalis were reduced to few peaks. Within the carbohydrate region (50-85 ppm) many of the same peaks were noted in all species. However each species had a different ratio between peaks as well as individual intensity variances of specific peaks. An example was

peak S (70.4 ppm) which was a major peak in C. albicans but was not significantly present in any of the other species. Therefore it was quite possible to distinguish species with NMR.

To answer the second question of strain differences and identification, 24 strains of C. albicans were incubated as specified above and then the spectra accumulated with the NMR. Three major groups of spectra were identified. Nineteen C. albicans showed type 1 spectra, 5 were type 2 spectra and 1 strain belonged to type 3. Within the groups no identification of strains was possible because of similar peaks in all spectra and the range of intensities of any one peak obtained from different spectra of the same strain of C. albicans. However, the fact that there are 2 distinct types did lead to the possibility of these groups being related to the antigenic groups A and B of isolates of C. albicans described by Hasenclever, et al (27, 28, 29).

In order to answer the question that these 3 groups were in fact the A and B serotypes of C. albicans, 4 known serotyped organisms were obtained from the American Type Culture Collection (ATCC). However when the ATCC strains were run in the NMR under standard conditions, the B's looked like type 3, and the A's didn't fit any

category found so far. Therefore the A's were put into a type 4 category until further investigation can be done. One possible explanation for the ATCC strains being very different from all other strains of C. albicans looked at could be length of storage. The ATCC cultures had been lyophilized, some for as long as 10 years before we received them. Some early experiments with fresh and lyophilized cells showed that the spectra from lyophilized cells do show changes compared to fresh cells. However, since the cells were passed 4 times in SAAMF broth before any spectra were run and multiple repetitions have given the same spectra, it's unknown whether lyophilization was the cause of the different spectra for the ATCC cultures. It may be that there are many more types of spectra than we have been able to detect with the small group of organisms we have looked at and further investigation would reveal other similar type 4 organisms.

The third question to answer was what environmental and physical factors that affect the growth of cells will effect the NMR spectra obtained. For the growth phase studies a type 1 and 2 C. albicans were incubated for 4, 7, 20 and 24 h in SAAMF and the NMR spectra compared (Figure 7,8). The C. albicans type 2 was usually characterized by 3 major peaks in the carbohydrate region

(50-85 ppm) in a 24 h spectrum. However the early (4, 7 h) spectra include many more peaks in the carbohydrate region as well as peaks B (27 ppm) and D (33.2 ppm) in the aliphatic region that were not present in the later spectra. The C. albicans type 1 spectra had the same peaks at 7, 20 and 24 h, although the ratio of peak heights differed. All of the changes in the spectra can probably be explained by the different metabolism occurring in the cells at different times in the growth of the cells. Since the NMR spectra are combinations of carbon peaks that relate to the compounds within the cell, the spectra directly reflect the metabolism of the cells.

A difference in metabolic pathways was also reflected in the media studies. Three different media based on different nutritional sources were used to grow a C. albicans type 1 (Figure 9). Although the 3 spectra obtained were similar, the carbohydrate region (50-85 ppm) of each had different relative peak heights, as well as some differing peaks. Therefore each spectrum apparently reflected the different nutritional sources and metabolism of the organism in that medium.

Temperature had the most interesting and drastic effect on the cells and therefore the NMR spectra (Figure

10). When the C. albicans type 1 cells were lowered from an incubation temperature of 37°C to 28°C the spectra lost many of the carbohydrate region (50-85 ppm) peaks. The loss of peaks in the 28°C spectrum resulted in a spectrum that was indistinguishable from a type 2 C. albicans spectrum, with the 3 major peaks in the carbohydrate region and reduced peaks in the aliphatic region (10-35 ppm).

The last set of questions considered dealt with reproducibility of spectra from cells passed continuously in culture, passed in animals and mixed with other strains. The first question of continuous passage, relates to the problems in the dimorphism experiments. The cells for experiments were maintained in continuous culture by transferring every 48 h over a period of months. It was noted with the dimorphism experiments that the spectrum of the strain used in the studies was beginning to change over the course of the experiments resulting in a more complex spectrum. It was postulated that the continuous transferring could be causing the problems in the instability of the culture and ultimately the spectra. Therefore several strains of C. albicans were passed continuously in SAAMF over several months and periodic spectra were obtained. It was shown that the spectra do change over time through the addition of extra

peaks to the spectra, primarily in the carbohydrate region (50-85 ppm). This same phenomenon of extra peak addition was also seen in the strains that were mixed together and in the strains that were passed through mice. Even though in both of these latter experiments the strains were passed at least 2 times in SAAMF after passage and before NMR.

NMR does have an important place in biological applications and ^{13}C NMR is a good addition to the standard use of ^{31}P . At this point, however NMR is primarily useful for simple problems under very controlled conditions. However, with more study perhaps the more difficult problems of dimorphism can be approached with confidence.

LITERATURE CITED

LITERATURE CITED

1. Abraham, R.J. and P. Loftus. (1978). Proton and Carbon-13 NMR Spectroscopy: An Integrated Approach. Heyden. Philadelphia.
2. Alger, J.R., J.A. den Hollander and R.G. Shulman. (1982). In vivo phosphorus-31 nuclear magnetic resonance saturation transfer studies of adenosinetriphosphate kinetics in Saccharomyces cerevisiae. Biochemistry 21: 2957-2963.
3. Barton, J.K., J.A. den Hollander, J.J. Hopfield and R.G. Shulman. (1982). ¹³C Nuclear magnetic resonance study of trehalose mobilization in yeast spores. J. Bacteriol. 151: 177-185.
4. Bedell, G.W. and D.R. Soll. (1979). Effects of low concentrations of zinc on the growth and dimorphism of Candida albicans: Evidence for zinc-resistant and -sensitive pathways for mycelium formation. Infect. Immun. 26: 348-354.
5. Bhattacharya, A., S. Banerjee and A. Datta. (1974). Regulation of N-acetylglucosamine kinase synthesis in yeast. Biochem. Biophys. Acta. 374: 384-391.
6. Brown, L.A. and W.L. Chaffin. (1981). Differential expression of cytoplasmic proteins during yeast bud and germ tube formation in Candida albicans. Can. J. Microbiol. 27: 580-585.
7. Brown, T.R., K. Ugurbil and R.G. Shulman. (1977). ³¹P Nuclear magnetic resonance measurements of ATPase kinetics in aerobic Escherichia coli cells. Proc. Natl. Acad. Sci. 74: 5551-5553.
8. Brummel, M. and D.R. Soll. (1982). The temporal regulation of protein synthesis during synchronous bud or mycelium formation in the dimorphic yeast Candida albicans. Developmental Biol. 89: 211-224.
9. Cassone, A., G. Carpinelli, L. Angiolella, G. Maddaluno, and F. Podo. (1983). ³¹P Nuclear magnetic resonance study of growth and dimorphic transition in Candida albicans. J. Gen. Microbiol. 129: 1569-1575.

10. Chaffin, W.L. and S.J. Sogin. (1976). Germ tube formation from zonal rotor fractions of Candida albicans. J. Bacteriol. 126: 771-776.
11. Chattaway, F.W. and M.R. Holmes. (1968). Cell wall composition of the mycelial and blastospore forms of Candida albicans. J. Gen. Microbiol. 51: 367-376.
12. Chattaway, F.W., R. Bishop, M.R. Holmes and F.C. Odds. (1973). Enzyme activities associated with carbohydrate synthesis and breakdown in the yeast and mycelial forms of Candida albicans. J. Gen. Microbiol. 75: 97-109.
13. Chattaway, F.W., P.R. Wheeler and J. O'Reilly. (1980). Purification and properties of peptides which induce germination of blastospores of Candida albicans. J. Gen. Microbiol. 120: 431-437.
14. Chattaway, F.W., P.R. Wheeler and J. O'Reilly. (1981). Involvement of adenosine 3':5'-cyclic monophosphate in the germination of blastospores of Candida albicans. J. Gen. Microbiol. 123: 233-240.
15. Cutler, J.E. (1976). Acute systemic candidiasis in normal and congenitally thymic-deficient (nude) mice. J. Reticuloendothel. Soc. 19: 121-124.
16. Dabrowa, N., S.S.S. Taxer and D.H. Howard. (1976). Germination of Candida albicans induced by proline. Infect. Immun. 13: 830-835.
17. Dabrowa, N. and D.H. Howard. (1981). Proline uptake in Candida albicans. J. Gen. Microbiol. 127: 391-397.
18. Den Hollander, J.A., T.R. Brown, K. Ugurbil and R.G. Shulman. (1979). ¹³C nuclear magnetic resonance studies of anaerobic glycolysis in suspensions of yeast cells. Proc. Natl. Acad. Sci. 76: 6096-6100.
19. Diamond, R.D., R. Krzesicki and W. Jao. (1978). Damage to pseudohyphal forms of Candida albicans by neutrophils in the absence of serum in vitro. J. Clin. Invest. 61: 349-359.

20. Diamond, R.D. and R. Krzesicki (1978). Mechanisms of attachment of neutrophils to Candida albicans pseudohyphae in the absence of serum, and of subsequent damage to pseudohyphae by microbicidal processes of neutrophils in vitro. J. Clin. Invest. 61: 360-369.
21. Eakin, R.T., L.O. Morgan, C.T. Gregg and N.A. Matwiyoff. (1972). Carbon-13 nuclear magnetic resonance spectroscopy of living cells and their metabolism of a specifically labelled ¹³C substrate. FEBS Letters 28: 259-264.
22. Elorza, M.V., H. Rico, D. Gozalbo and R. Sentandreu. (1983). Cell wall composition and protoplast regeneration in Candida albicans. Antonie van Leeuwenhoek 49: 457-469.
23. Emmons, C.W., C.H. Binford, J.P. Utz and K.J. Kwon-chung. (1977). Medical Mycology, 3rd edition. Lea and Febiger. Philadelphia.
24. Evans, E.G.V., F.C. Odds, M.D. Richardson and K.T. Holland. (1975). Optimum conditions for initiation of filamentation in Candida albicans. Can. J. Microbiol. 21: 338-342.
25. Findly, R.C., R.J. Gillies and R.G. Shulman. (1983). In vivo phosphorus-31 nuclear magnetic resonance reveals lowered ATP during heat shock of Tetrahymena. Science 219: 1223-1225.
26. Gillies, R.J. and A.G. Benoit. (1983). NMR analysis of a cell division cycle mutant of Saccharomyces cerevisiae. Biochem. Biophys. Acta. 762: 466-470.
27. Hasenclever, H.F. and W.O. Mitchell. (1960). Antigenic relationships of Torulopsis glabrata and seven species of the genus Candida. J. Bacteriol. 79: 677-681.
28. Hasenclever, H.F. and W.O. Mitchell. (1961). Antigenic studies of Candida: I. Observation of two antigenic groups in Candida albicans. J. Bacteriol. 82: 570-573.

29. Hasenclever, H.F., W.O. Mitchell and J. Loewe. (1961). Antigenic studies of Candida: II. Antigenic relation of Candida albicans group A and group B to Candida stellatoidea and Candida tropicalis. J. Bacteriol. 82: 574-577.
30. Hazen, K.C. and J.E. Cutler. (1979). Autoregulation of germ tube formation by Candida albicans. Infect. Immun. 24:661-666.
31. Hazen, K.C. and J.E. Cutler. (1983). Effect of cobalt and morphogenic autoregulatory substance (MARS) on morphogenesis of Candida albicans. Exp. Mycology 7: 182-187.
32. Hazen, K.C. and J.E. Cutler. (1983). Isolation and purification of morphogenic autoregulatory substance produced by Candida albicans. J. Biochem. 94: 777-783.
33. Hedden, D.M. and J.D. Buck. (1980). A reemphasis-germ tubes diagnostic for Candida albicans have no constrictions. Mycopathologia 70: 95-101.
34. Hill, D.W. and L.P. Gebhardt. (1956). Morphological transformation of Candida albicans in tissues of mice. Proc. Soc. Exp. Biol. Med. 92: 640-644.
35. Hrmova, M. and L. Drobica. (1981). Induction of mycelial type of development in Candida albicans by low glucose concentration. Mycopathologia 76: 83-96.
36. Hrmova, M. and L. Drobica. (1982). Induction of mycelial type of development in Candida albicans by the antibiotic monorden and N-acetyl-D-glucosamine. Mycopathologia 79: 55-64.
37. Hoeprich, P.D. (1983). Candidosis. In Infectious Diseases, 3rd edition, P.D. Hoeprich (editor). Harper and Row, Hagerstown, Maryland. Pp. 436-450
38. Jacobson, L., M. Halmann and J. Yariv. (1982). The molecular composition of the volutin granule of yeast. Biochem. J. 201: 473-479.

39. Kainosho, M., K. Ajisaka and H. Nakazawa. (1977). In situ analysis of the microbial fermentation process by natural abundance ^{13}C and ^{31}P NMR spectroscopy. Production of adenosine-5'-triphosphate from adenosine. *FEBS Letters* 80: 385-389.
40. Land, G.A., W.C. McDonald, R.L. Stjernholm and L. Friedman. (1975). Factors affecting filamentation in Candida albicans: Relationship of the uptake and distribution of proline to morphogenesis. *Infect. Immun.* 11: 1014-1023.
41. Larsen, A.D. and P.S. Sypherd. (1974). Cyclic adenosine 3',5'-monophosphate and morphogenesis in Mucor racemosus. *J. Bacteriol.* 117: 432-438.
42. Levy, G.C., R.L. Lichter and G.L. Nelson. (1980). Carbon-13 Nuclear Magnetic Resonance Spectroscopy. John Wiley and Sons. New York.
43. Luria, D.B. and R.G. Brayton. (1964). Behavior of Candida cells within leukocytes. *Proc. Soc. Exp. Biol. Med.* 115: 93-98.
44. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193: 265-275.
45. Manning, M. and T.G. Mitchell. (1980). Morphogenesis of Candida albicans and cytoplasmic proteins associated with differences in morphology, strain, or temperature. *J. Bacteriol.* 144: 258-273.
46. Manning, M. and T.G. Mitchell. (1980). Analysis of cytoplasmic antigens of the yeast and mycelial phases of Candida albicans by two-dimensional electrophoresis. *Infect. Immun.* 30: 484-495.
47. Maresca, B., G. Medoff, D. Schlessinger, G.S. Kobayashi and J. Medoff. (1977). Regulation of dimorphism in the pathogenic fungus Histoplasma capsulatum. *Nature* 266: 447-448.
48. Matsunaga, T., A. Okubo, S. Yamazaki and S. Toda. (1980). Natural abundance ^{13}C nuclear magnetic resonance spectra of intact fungal mycelia. *Agric. Biol. Chem.* 44: 2757-2759.

49. Mattia, E. and A. Cassone. (1979). Inducibility of germ-tube formation in Candida albicans at different phases of yeast growth. J. Gen. Microbiol. 113: 439-442.
50. McClary, D.O. (1952). Factors affecting the morphology of Candida albicans. Annals of the Missouri Botanical Garden 39: 137-163.
51. Medoff, J., E. Jacobson and G. Medoff. (1981). Regulation of dimorphism in Histoplasma capsulatum by cyclic adenosine 3',5'-monophosphate. J. Bacteriol. 145: 1452-1455.
52. Nickerson, W.J. (1963). Symposium on biochemical bases of morphogenesis in fungi. Bacteriol. Rev. 27: 305-324.
53. Niimi, M., K. Niimi, J. Tokunaga and H. Nakayama. (1980). Changes in cyclic nucleotide levels and dimorphic transition in Candida albicans. J. Bacteriol. 142: 1010-1014.
54. Pall, M.L. (1981). Adenosine 3',5'-phosphate in fungi. Microbiol. Rev. 45: 462-480.
55. Paznokas, J.L. and P.S. Sypherd. (1975). Respiratory capacity, cyclic adenosine 3',5'-monophosphate, and morphogenesis of Mucor racemosus. J. Bacteriol. 124: 134-139.
56. Pugh, D. and R.A. Cawson. (1979). The induction of germ tubes in Candida albicans by an intrinsic factor. Microbios 24: 73-79.
57. Rebora, A., R.R. Marples and A.M. Kligman. (1973). Experimental infection with Candida albicans. Arch. Dermatol. 108: 69-73.
58. Rinaldi, M.G. (1982). Use of potato flakes agar in clinical mycology. J. Clin. Microbiol. 15: 1159-1160.
59. Rippon, J.W. (1982). Candidiasis and the pathogenic yeasts. In Medical Mycology: The Pathogenic Fungi and the Pathogenic Actinomycetes. W.B. Saunders Co., Philadelphia. Pp. 484-531.

60. Roberts, J.K.M. and O. Jardetzky. (1981). Monitoring of cellular metabolism by NMR. *Biochem. Biophys. Acta.* 639: 53-76.
61. Schwartz, D.S. and H.W. Larsh. (1982). Comparative activities of glycolytic enzymes in yeast and mycelial forms of Candida albicans. *Mycopathologia* 78: 93-98.
62. Shepherd, M.G., H.M. Ghazali and P.A. Sullivan. (1980). N-acetyl-D-glucosamine kinase and germ-tube formation in Candida albicans. *Exp. Mycology* 4: 147-159.
63. Shepherd, M.G., Chiew Yoke Yin, S.P. Ram and P.A. Sullivan. (1980). Germ tube induction in Candida albicans. *Can. J. Microbiol.* 26: 21-26.
64. Shulman, R.G., T.R. Brown, K. Ugurbil, S. Ogawa, S.M. Cohen and J.A. den Hollander. (1979). Cellular applications of ^{31}P and ^{13}C nuclear magnetic resonance. *Science* 205: 160-166.
65. Shulman, R.G. (1983). NMR spectroscopy of living cells. *Scientific Am.* 248: 86-93.
66. Simonetti, N., V. Strippoli and A. Cassone. (1974). Yeast-mycelial conversion induced by N-acetyl-D-glucosamine in Candida albicans. *Nature* 250: 344-346.
67. Skoog, D.A. and D.M. West. (1980). Principles of instrumental analysis. 2nd edition. Saunders College. Philadelphia.
68. Soll, D.R., G.W. Bedell and M. Brummel. (1981). Zinc and the regulation of growth and phenotype in the infectious yeast Candida albicans. *Infect. Immun.* 32: 1139-1147.
69. Sullivan, P.A. and M.G. Shepherd. (1982). Gratuitous induction by N-acetylmannosamine of germ tube formation and enzymes for N-acetylglucosamine utilization in Candida albicans. *J. Bacteriol.* 151: 1118-1122.

70. Sundaram, S., P.A. Sullivan and M.G. Shepherd. (1981). Changes in lipid composition during starvation and germ-tube formation in Candida albicans. Exp. Mycology 5: 140-147.
71. Syverson, R.E., H.R. Buckley and C.C. Campbell. (1975). Cytoplasmic antigens unique to the mycelial or yeast phase of Candida albicans. Infect. Immun. 12: 1184-1188.
72. Thevelein, J.M., J.A. den Hollander and R.G. Shulman. (1982). Changes in the activity and properties of trehalose during early germination of yeast ascospores: Correlation with trehalose breakdown as studied by in vivo ¹³C NMR. Proc. Natl. Acad. Sci. 79: 3503-3507.
73. Thorner, J. (1982). An essential role for cyclic AMP in growth control: The case for yeast. Cell 30: 5-6.
74. Trevillyan, J.M. and M.L. Pall. (1979). Control of cyclic adenosine 3',5'-monophosphate levels by depolarizing agents in fungi. J. Bacteriol. 138: 397-403.
75. Vaughn, V.J. and E.D. Weinberg. (1978). Candida albicans dimorphism and virulence: Role of copper. Mycopathologia 64: 39-42.
76. Wain, W.H., M.F. Price and R.A. Cawson. (1975). A reevaluation of the effect of cysteine on Candida albicans. Sabouraudia 13: 74-82.
77. Wain, W.H., A.R. Brayton and R.A. Cawson. (1976). Variations in the response to N-acetyl-D-glucosamine by isolates of Candida albicans. Mycopathologia 58: 27-29.
78. Winner, H.I. (1969). The transition from commensalism to parasitism. Br. J. Derm. 81: 62-68.
79. Wolter, J.R. (1962). Endogenous fungus endophthalmitis. Arch. Ophthalmol. 68: 337-340.

80. Wurster, B., F. Bek and U. Butz. (1981). Folic acid and pterin deaminases in Dictyostelium discoideum: Kinetic properties and regulation by folic acid, pterin, and adenosine 3',5'-phosphate. J. Bacteriol. 148: 183-192.
81. Yamaguchi, H. (1975). Control of dimorphism in Candida albicans by zinc: Effect on cell morphology and composition. J. Gen. Microbiol. 86: 370-372.

APPENDICES

Appendix I

Techniques of Measuring cAMP

The techniques used to measure cAMP, in the experiments presented, were not arrived at easily. The procedures for the experiments can be divided into 4 parts. These are: preparation of C. albicans cells; cAMP extraction; cAMP assay; and protein determination. Erratic results were obtained throughout the experimentation until the final protocols were achieved.

Problems in the cell preparation first occurred when the C. albicans strain 9938, used for experimentation continually failed to germinate (see appendix II). Therefore strain 394 was used to replace 9938. Duplicate and later triplicate flasks of each time period were grown to test for cAMP content. Finally it was determined that duplicate flasks and duplicate samples from each flask was the best way to check for consistency of results. The shaking incubator used was a water bath rather than a hot air incubator for a more constant temperature during the experiments.

Changes in protocol occurred the most often in the cAMP extraction procedures. The first protocol tried was based on work done by Paznokas and Sypherd (17). First,

cells were collected by membrane filtration (100 mm, 0.45 μ m) and the filter was placed in 2.5 ml of ice-cold 0.1 N HCl. The samples were subjected to 3 cycles of freezing (methanol: -50°C) and then thawing (water bath: 37°C). Filters were removed and the samples centrifuged at 1500 rpm for 10 min. The supernatant fluid from the sample was removed and flash evaporated until dry, then stored frozen.

The first problem area was the filtration of samples to add to the HCl. This was a slow process with some loss of material. Centrifugation, an easier process for separating cells was then tried. However, it was learned that levels of cAMP can change very quickly, within 10-30 sec. Therefore filtration was tried again but with 50 ml syringes and 25 mm filters. This proved to be the fastest (under 30 sec from incubator to HCl) and with washing to improve recovery, the best method.

Freezing for freeze-thawing was first done in 12 ml centrifuge tubes in a -50°C methanol bath. This was later replaced with a better method of placing the filters in 10 ml beakers of HCl and freezing in a dry ice: ethanol (-70°C) bath. The method of flash evaporation of the samples in the first experiments was replaced by evaporation of the samples in a 60°C water bath under N_2 . The samples must be evaporated to complete dryness,

because any HCl in the tubes will affect the final pH after reconstitution. Since the acetate buffer has little buffering capacity, the lowered pH can lead to false results of high levels of cAMP. Evaporation overnight and then heating for 2 h in a low temperature oven was then used.

Another method that used glass beads to break cells (8) was tried instead of freeze-thawing. However hyphae did not break well so a third protocol was tried. The new protocol was based on work done by Niimi, et al. (14). Several major changes from the Paznokas and Sypherd (17) protocol were used. First 6% TCA (made fresh every experiment) instead of 0.1 N HCl was used. After 5 cycles of freeze-thawing, the cells were extracted with diethyl-ether to remove the TCA and then bubbled with N₂ to remove the ether. The sample was then centrifuged at 1500 x g for 15 min and the supernatant fluid was lyophilized rather than evaporated. Aluminum oxide and Dowex (1x8, 20-50 mesh) columns (13) used by Niimi, et al. (14) were tried to further purify the supernatant fluid but no significant increase in yield of cAMP was obtained.

The cAMP assay kit used to determine the cAMP content was another source of erratic results. First a ³H

competitive binding assay (3, 5, 6) from Amersham (Arlington Heights, Illinois) was employed. Experiments were done to show that a linear increase in cell numbers did not produce a linear increase in cAMP. Erratic results between identical samples and quenching of random samples led to the switch to another kit. An ^{125}I RIA kit (7, 22) from New England Nuclear (Boston, Mass.) worked well for all further experiments.

Final problems occurred in the determination of protein concentrations. Some methods tried were the Biorad (Richmond, CA) protein assay kit (2, 19, 24) tested on the supernatant fluids from both 1.0 N NaOH boiled and glass bead broken cell pellets and also dry weights of pellets. None of these techniques were sensitive or reliable enough. Therefore pellets were boiled for 20 min in 1.0 N NaOH, centrifuged at 1500xg for 15 min and the supernatant fluid was tested with the Lowry method (12).

Appendix II

Problems With Dimorphism

Among strains of C. albicans there is a range of abilities to germinate as well as the length of time that any strain will germinate. The percent germination of strains will be high for varying lengths of time from several months to several years. Then germination will decline rapidly to a very low level. The cause of this phenomenon is unknown but the occurrence is common.

Strains kept in GYEP in continuous passage and transferred every 48 h, were used as starters for dimorphism experiments. There was a change in NMR spectra of the strains that were passed continuously in SAAMF. Background noise increased and several additional peaks were observed. It's unknown if these changes are related to the problems encountered in dimorphism.

Appendix III

Enzyme Studies

Several enzyme protocols were worked out in order to look at enzyme levels in C. albicans Y and M forms. Because of problems in C. albicans strain 394 germinating ability (Appendix II), the studies were not completed. The final protocols are given.

Ornithine decarboxylase is the first enzyme in the biosynthesis pathway of polyamines. It is associated with increases in protein and RNA synthesis and there is some evidence that it's regulation is linked to cAMP (1, 4, 20, 23). The protocol used to look at this enzyme is based on the work of Inderlied, Cihlar and Sypherd (9) on Mucor. These researchers found a 30-50 fold increase in activity in Y-->M conversion.

The first part of this protocol involves setting up a CO₂ trap to capture the ¹⁴CO₂ evolved in the reaction. A modification of Sissons (21) trap was used (Figure 12). A polypropylene T-junction was attached via rubber tubing to a 10 x 75 mm test tube. The opposite end of the junction was attached by rubber tubing to a pasteur pipette that extends down into the test tube. Tubing hooked up to an air flow was attached to one end of the pipette. The other end of the pipette had small tubing

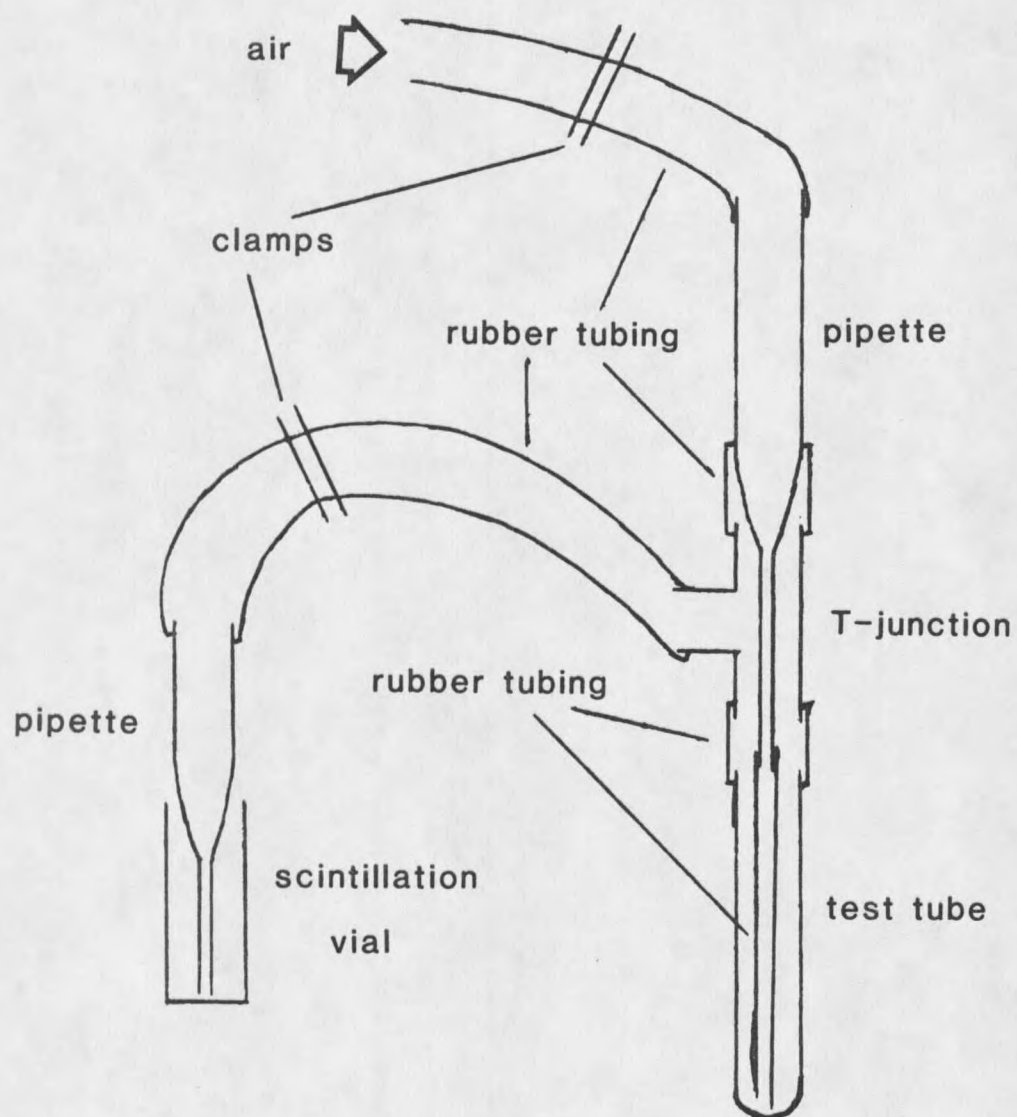


Figure 12. CO₂ trap

attached so that the tip won't break and the flow of air can reach down into the sample. The side arm of the T-junction was attached by tubing to another pasteur pipette that extends into a glass scintillation vial that contains Carbosorb (Packard, Downers Grove, ILL) an organic absorber.

Preparation of the cells for the assay was done according to the cAMP assay cell preparation method in the Materials and Methods section. The only modification was that approximately 10^{10} cells were necessary to detect the enzyme. Therefore more flasks must be run. The cells obtained were washed and suspended in 5ml of 0.1 M potassium phosphate buffer (pH 7.3) with 2mM dithiothreitol (DTT), 1 mM EDTA and 0.2 mM pyridoxal 5' phosphate. Ten grams of glass beads (0.45-0.5 mm) were added and cells broken and centrifuged (8). The supernatant fluid was frozen until needed for the enzyme assay.

The assay was begun by adding 50 ul of the supernatant fluid to the 10 x 75 mm test tube in a 30°C water bath. Then 50 ul of assay mix [DTT + EDTA + pyridoxal 5' phosphate (same concentrations as the breakage buffer) + 0.5 mM ornithine + 50 nCi ^{14}C -ornithine] was added to the tube. The tube was quickly vortexed, the CO_2 trap added, clamps closed and incubated

for 30 min. Just prior to the end of the incubation, 0.5 ml of Carbosorb was added to the scintillation vial. At the end of the incubation, 0.1 ml 2 M HClO_4 was injected with a 1 cc syringe directly into the tube, air was hooked up and all clamps opened for 5 min. After 5 min, 4 ml of scintillation cocktail [1.0 g PPO: 80 ml methoxy ethanol (J.T. Baker Chemical Company, Phillipsburg, NJ): 120 ml toluene (Research Products International Corporation, Elk Grove, ILL)] was added to the vial. The vial was capped and counted.

The second enzyme to study was glutamate dehydrogenase which has been looked at in other fungi (10, 11, 15, 16). The protocol used was based on Peters and Sypherd (18) work on Mucor. These researchers found the NAD dependent activity an order of magnitude higher in the M than Y form of Mucor.

The cell preparation was the same as for the ornithine decarboxylase enzyme. Approximately 10^{10} cells were needed and a 0.2 M potassium phosphate buffer (pH 8.0) was used to suspend cells for breakage. Each enzyme sample was adjusted to contain a 0.001 M concentration of 2-mercaptoethanol.

The assay was run in a Gilford spectrometer at 340 nm, visible light with a blue filter. To a cuvette was

added 100 ul of sample, 100 ul buffer, 100 ul 2 M NH_4Cl in buffer, 100 ul 0.3 M α -ketoglutarate in buffer and 500 ul of deionized water. To start the reaction 100 ul of 1.25 mM NADH or NADPH in buffer was added, mixed by inversion and read every 30 sec for 10 min.

LITERATURE CITED IN APPENDICES

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1. Bachrach, U. (1975). Cyclic AMP-mediated induction of ornithine decarboxylase of glioma and neuroblastoma cells. *Proc. Nat. Acad. Sci.* 9: 3087-3091.
2. Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.
3. Brown, B.L., J.D.M. Albano, R.P. Ekins and A.M. Sgherzi. (1971). A simple and sensitive saturation assay method for the measurement of adenosine 3':5'-cyclic monophosphate. *Biochem. J.* 121: 561-562.
4. Byus, C.V. and D.H. Russell. (1975). Ornithine decarboxylase activity: Control by cyclic nucleotides. *Science* 187: 650-652.
5. Gilman, A.G. (1970). A protein binding assay for adenosine 3':5'-cyclic monophosphate. *Proc. Nat. Acad. Sci.* 67: 305-312.
6. Gilman, A.G. and F. Murad. Assay of cyclic nucleotides by receptor protein binding displacement. *Methods of Enzymology* 38: 49-57.
7. Hatch, G.E., W.K. Nichols and H.R. Hill. (1978). A simplified procedure for cyclic nucleotide radioimmunoassay and its applications to human blood leukocytes. *J. Immunol. Methods* 19: 301-308.
8. Hazen, K.C. and J.E. Cutler. (1982). Optimal conditions for breaking medically important yeasts by an inexpensive and simple method. *Mycopathologia* 80: 113-116.
9. Inderlied, C.B., R.L. Cihlar and P.S. Sypherd. (1980). Regulation of ornithine decarboxylase during morphogenesis of Mucor racemosus. *J. Bacteriol.* 141: 699-706.
10. Kapoor, M. and A.K. Grover. (1970). Catabolite-controlled regulation of glutamate dehydrogenases of Neurospora crassa. *Can. J. Microbiol.* 16: 33-40.

11. Kinghorn, J.R. and J.A. Pateman. (1973). NAD and NADP L-glutamate dehydrogenase activity and ammonium regulation in Aspergillus nidulans. J. Gen. Microbiol. 78: 39-46.
12. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall. (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.
13. Mao, C.C. and A. Guidotti. (1974). Simultaneous isolation of adenosine 3',5'-cyclic monophosphate (cAMP) and guanosine 3',5'-cyclic monophosphate (cGMP) in small tissue samples. Anal. Biochem. 59: 63-68.
14. Niimi, M., K. Niimi, J. Tokunaga and H. Nakayama. (1980). Changes in cyclic nucleotide levels and dimorphic transition in Candida albicans. J. Bacteriol. 142: 1010-1014.
15. Nunez de Castro, I., M. Ugarte, A. Cano and F. Mayor. (1970). Effect of glucose, galactose, and different nitrogen-sources on the activity of yeast glutamate dehydrogenase (NAD and NADP-linked) from normal strain and impaired respiration mutant. Eur. J. Biochem. 16: 567-570.
16. Pateman, J.A. (1969). Regulation of synthesis of glutamate dehydrogenase and glutamine synthetase in micro-organisms. Biochem. J. 115: 769-775.
17. Paznokas, J.L. and P.S. Sypherd. (1975). Respiratory capacity, cyclic adenosine 3',5'-monophosphate, and morphogenesis of Mucor racemosus. J. Bacteriol. 124: 134-139.
18. Peters, J. and P.S. Sypherd. (1979). Morphology-associated expression of nicotinamide adenine dinucleotide-dependent glutamate dehydrogenase in Mucor racemosus. J Bacteriol. 137: 1134-1139.
19. Pierce, J. and C.H. Suelter. (1977). An evaluation of the coomassie brilliant blue G-250 dye-binding method for quantitative protein determination. Anal. Biochem. 81: 478-480.

20. Russell, D.H. and P.J. Stambrook. (1975). Cell cycle specific fluctuations in adenosine 3':5'-cyclic monophosphate and polyamines of chinese hamster cells. Proc. Nat. Acad. Sci. 72: 1482-1486.
21. Sissons, C.H. (1976). Improved technique for accurate and convenient assay of biological reactions liberating $^{14}\text{CO}_2$. Anal. Biochem. 70: 454-462.
22. Steiner, A.L., D.M. Kipnis, R. Utiger and C. Parker. (1969). Radioimmunoassay for the measurement of adenosine 3',5'-cyclic phosphate. Biochem. 64: 367-373.
23. Theoharides, T.C. and Z.N. Canelakis. (1975). Spermine inhibits induction of ornithine decarboxylase by cyclic AMP but not dexamethasone in rat hepatoma cells. Nature 255: 733-734.
24. Van Kley, H. and S.M. Hale. (1977). Assay for protein by dye binding. Anal. Biochem. 81: 485-487.

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