Nuclear Magnetic Resonance Studies of the Arabinitol Metabolism and Classification of Candida Albicans
by MARK E ANDERSON

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry
Montana State University
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Abstract:
Carbon-13 nuclear magnetic resonance spectroscopy (NMR) has been increasingly applied to problems of biochemical interest. In this thesis, natural abundance C-13 NMR was used to guide an enriched C-13 study of the arabinitol metabolism of the yeast, Candida albicans. It also proved possible to classify strains of C. albicans into groups based upon their natural abundance C-13 NMR spectra.

C. albicans is an opportunistic, pathogenic yeast. In clinical and laboratory studies, detection of arabinitol has been shown to be useful. Natural abundance spectra showed that different strains of C. albicans had different internal levels of arabinitol. The cell stores of arabinitol were shown to be influenced by changes in the salinity of the growth medium and growth temperature.

These three observations guided the design of the experiments using l-C-13- and 2-C-13-glucose and l-C-13-xylose. 5 x 10^7 yeast cells/ml were suspended in yeast extract-peptone supplemented with lOmg of labeled glucose or xylose. Production of arabinitol, glycerol and ethanol were monitored using a Bruker WM-250 operating at 62.89 MHz. It was possible to determine the routes used to produce the glycerol and arabinitol by analyzing the label scrambling patterns.

The classification work was based upon the C-13 natural abundance spectra from twenty three strains of C. albicans. Two ml of cell slurry (10^10 cells/ml) were used to acquire the spectra in less than half an hour. The difference in the stores of trehalose, arabinitol and glutamate were used as the basis for classification.

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A thesis submitted in partial fulfillment
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APPROVAL

of a thesis submitted by

Mark Edward Anderson

This thesis has been read by me and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

Carbon-13 nuclear magnetic resonance spectroscopy (NMR) has been increasingly applied to problems of biochemical interest. In this thesis, natural abundance C-13 NMR was used to guide an enriched C-13 study of the arabinitol metabolism of the yeast, Candida albicans. It also proved possible to classify strains of C. albicans into groups based upon their natural abundance C-13 NMR spectra.

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The classification work was based upon the C-13 natural abundance spectra from twenty three strains of C. albicans. Two ml of cell slurry (10^{10} cells/ml) were used to acquire the spectra in less than half an hour. The difference in the stores of trehalose, arabinitol and glutamate were used as the basis for classification.

INTRODUCTION

Since the introduction of Fourier-transform spectrometers in the early 1970's, nuclear magnetic resonance spectroscopy (NMR) has been applied increasingly to biological and biochemical problems. The greatest advantage that NMR offers over the traditional methods, such as radiolabeling and enzyme assays, is that it is a non-invasive technique. It is possible to monitor the active metabolism of a live specimen from inside the spectrometer. A second advantage of NMR relates to the type of information obtained from the experiments. The spectra obtained can lead to the unambiguous identification of a compound and its concentration inside of the organism.

The non-invasive aspect of NMR studies of organisms is the advantage that is most often cited in justifying a series of experiments using a spectrometer. In studies in which cells are disrupted, it is difficult, and can be impossible, to determine if a particular observation is in fact what has occurred in the organism, or if what is observed is an artifact of the extraction. Unstable chemical intermediates can be destroyed by the often harsh conditions used to stop further metabolic activity. Because the organisms are not extracted during the NMR

experiment, unstable intermediates are not exposed to a possibly destablizing environment.

The NMR technique relies upon the existence of atomic nuclei that resonate at a given radiofrequency in a magnetic field. The resonant frequency of a given nucleus is determined by a combination of the applied magnetic field and the chemical environment of the nucleus. The applied field determines a frequency range and the chemical environment determines the exact frequency in that range. There are over 250 different nuclei available for analysis. The main nuclei accessible to the spectroscopist wanting to study metabolism are H-1, C-13, N-15 and P-31, but additional work has been done with such diverse nuclei as Na-23, and Cl-35 (1,2). Another factor that affects the use of NMR spectroscopy is the natural isotopic abundance of the nucleus of interest. Often, if the natural abundance is low, then the experiment is designed around the use of a labeled compound. If the natural abundance is high, then labeling is not necessary or can be difficult to do. All solution NMR techniques, regardless of the nucleus used, can detect only the smaller molecular weight molecules that are not immobilized due to some interaction. This can be an advantage or a limitation, depending upon the type of information desired by the investigators.

Proton NMR offers the highest degree of sensitivity, but it suffers from the ubiquitous presence of water in biological samples. The water resonance is broad enough and intense enough to obscure much that is chemically interesting. There are ways of getting around this, and recent work has shown that this may not always be a problem. Arus et al have looked at intact tissues using a selective water suppression pulse sequence (3,4). This method used a "jump and return" pulse sequence (5) and had the added advantage of allowing the detection of exchangeable protons. Serum, plasma, and urine analyses have been studied by proton NMR (6,7). Proton NMR has been used to follow the labeling of a carbon compound with C-13. The newer NMR techniques can be used for the acquisition of only those signals that arise from those protons attached to a labeled carbon (8). This can give a theoretical advantage in the signal-to-noise ratio (S/N) of 22:1, as well as the elimination of the water peak. A similar technique has been used to follow the incorporation of labeled acetate into its various products (9). The selectivity of the method allowed the identification of the products in the proton spectrum.

Nitrogen-15 NMR is accomplished predominantly through use of labeled compounds. N-15 has a natural isotopic abundance of 0.37%, and a sensitivity 1/1,000 that of proton. As expected, N-15 is used primarily to follow the

nitrogen metabolism of the organism. Kanamori et al investigated the effect that a nitrogen source had on the biosynthesis of glutamine and alanine in Neurospora crassa (10). N-15 NMR can be applied to other problems. Legerton et al used it to investigate the cytoplasmic and vascular pH in Neurospora crassa (11). It has also been used to make a direct measurement of the amount of crosslinking in the peptidoglycan of bacteria (12).

Phosphorus-31 NMR has the advantage of being 1/15th the sensitivity of H-1 and having 100% natural abundance. Although the 100% natural abundance precludes the use of normal isotopic labeling methods, some modern NMR techniques have been used to circumvent part of this limitation. It is possible to observe the biochemical activity associated with a particular phosphorous compound by saturating that resonance so that it cannot initially be detected, and then measuring the increase in the intensity of that resonance (13,14). The recovery of the intensity is a combination of the influx of outside nuclei and the relaxation of the original population. The difference of the two is an indication of the transfer of the phosphate groups from one population to the next population. Oxygen-17 has been used as a method of labeling a P-31 nucleus. This caused a shift in the phosphorus resonance that was used to differentiate between two possible phosphate substitution reactions (15).

P-31 NMR has been used to understand the roles of phosphorus-containing compounds in the energetics of an organism. den Hollander et al studied the effects of oxygen on glycolysis (16). Phosphorus NMR has been used to study the pH changes associated with several biologically important compounds (17), various changes occurring in blood cells (18) and the effect of light on photosynthetic bacteria (19).

Carbon-13 NMR has an advantage over the other methods of doing NMR studies because both labeled and unlabeled studies can be accomplished rather easily. Carbon-13 has a natural abundance of 1.1% and a sensitivity only 1/60 that of proton NMR. The low natural abundance means that a compound enriched to the 90%+ level in the desired position has almost two orders of magnitude greater intensity than background signals, concentrations being equal. Such a label can be followed as it passes from one intermediate to another during the metabolism of an organism. As with any labeling study, only those compounds that have both a sufficient concentration and are labeled will be detected. If the intermediate of interest does not accumulate to a level greater than the noise level, it will be missed by the experiment. If the starting material is not labeled in the proper site, then the intermediate(s) will not be labeled. Proper experimental design should avoid these obvious pitfalls, but then the investigator may miss the

unexpected. A natural abundance study does not suffer from the limitation of a misplaced label. By its very nature, all of the resonances of a compound are present. This is offset by the lack of sensitivity of the technique. The use of the newer, more sensitive spectrometers has partially overcome the sensitivity problem, but it still limits the utility of this technique when applied to biological investigations. To get around this disadvantage, it is possible to use a uniformly enriched substrate (20). The degree of labeling used is dependent upon the type of experiment. Some of the two-dimensional NMR techniques would benefit from the use of a 90%+ enrichment, but a standard one-dimensional spectrum may be overwhelmed by the interactions between neighboring C-13 nuclei. A useful level of uniform enrichment is approximately 20%. This minimizes the carbon-carbon interactions to 4% of the total number of nuclei.

C-13 NMR of biological systems has usually been accomplished through the use of labeled compounds. The analyses done using carbon-13 labeling are then analogous to those done with carbon-14. The additional advantage that NMR gives is the knowledge of the chemical structure of the compound. One of the first studies using C-13 NMR was done by Eakin et al (25). They used 1-C-13-glucose to study the anaerobic metabolism of Candida utilis. R. G. Shulman has done numerous studies using enriched

compounds to study metabolism. He and his co-workers have studied label scrambling in yeast glycolysis and trehalose production using 1-C-13-glucose and 6-C-13-glucose (21) and trehalose mobilization and utilization in yeast spores (22,23). Sacrificing the non-invasive aspect of NMR metabolic studies, Paalme, Olivson, and Vilu used the structural identification power of the technique to analyze the products of the photoassimilation and heterotrophic growth of Chlorobium thiosulfatophilum fed C-13 labeled carbon dioxide and acetate (24,25). Ogino et al studied the biosynthesis of aromatic compounds in Escherichia coli using 1-C-13-glucose (26). Since the normal concentrations of the aromatic amino acids in E. coli are below the detection limit for even a labeled C-13 study, they used mutant strains to enhance the production of these products in the pathways.

The alternative to enrichment, although not as well explored, is natural abundance spectroscopy. Natural abundance C-13 studies have become possible given the advances that high field NMR has made since the mid-1970's. The natural abundance spectra show all of the resonances due to small molecules given sufficient concentration in solution. This technique offers different information than that which is available from labeled C-13 NMR experiments. Although relatively few studies have been done using natural abundance, they have spanned a wide

range of biological systems. One of the first examples of natural abundance NMR of biological systems involved the production of adenosine-5'-triphosphate from adenosine in baker's yeast (27). In this study, Kainosho and co-workers used the presence of all of the spectroscopic peaks of the compounds present to properly identify trehalose. Trehalose had been previously observed in a study using enriched material, but was mistakenly identified as glucose-6-phosphate. R. Deslauriers et al studied the differentiation of amoeba by C-13 NMR (28). They noted the appearance of α,α-trehalose in the cells, which marked only the second time that disaccharide has been found in protozoa. One of the more intriguing studies involved the natural abundance analysis of attached grape berries (29). B. G. Coombe and G. P. Jones analyzed changes in a grape berry as it ripened over the course of three months, following the increasing concentrations of glucose and fructose and the decreasing concentrations of tartrate and malate. Lactic acid content of a frog muscle was quantified by use of natural abundance spectroscopy (30). This study showed good agreement between the NMR derived concentrations and those found by traditional, destructive methods. Additional systems studied by natural abundance spectroscopy include intact nerve tissues (31), intact muscle (32,33,34), excised rat livers (35,36) and intact rat livers (37,38,39), fruit pulps (40), live cestodes

(41), germinating soybean seeds (42), and various non-yeast fungi (43,44,45).

Definition of the Problem

The arabinitol metabolism of C. albicans was studied using natural abundance and isotopically enriched C-13 NMR spectroscopy. Other work was done to further the use of natural abundance C-13 NMR as a yeast classification technique.

Arabinitol Studies

Natural abundance spectra of C. albicans revealed an abundance of what was later identified as arabinitol. In clinical and laboratory studies the detection of arabinitol has been shown to be a possible indication of disseminated candidiasis (46,47,48,49,50,51,52). Not all cases of disseminated candidiasis, however, show positive results for the presence of arabinitol using the standard methods (56). It has been subsequently proposed that the stereoisomeric configuration of arabinitol can be used to detect disseminated candidiasis (58). Given the high internal levels of arabinitol in the yeast, I became intrigued with arabinitol production by C. albicans and attempted to elucidate some of the conditions that induced its production by the yeast.

The arabinitol metabolism of C. albicans was influenced by growth temperature, saline concentration, age of the culture and the strain of C. albicans used. Using these parameters to aid in the design of the experiments, I studied the production of arabinitol by C. albicans using labeled C-13 NMR. The yeast were fed one and two labeled glucose and one labeled xylose. The utilization of glucose and the accumulation of excretion products were followed. Samples of the excretion products and cell extracts were also analyzed. The results of these accumulations provided insight into the metabolic pathways used by C. albicans to produce arabinitol.

Natural Abundance Studies

The origin of this work is found in an attempt to apply natural abundance C-13 NMR spectroscopy to the problem of elucidating the chemical basis for the changes occurring in germinating cells of Candida albicans (65). C. albicans is an opportunistic pathogenic yeast that is responsible for a majority of nosocomial infections. Two morphological forms are associated with infection by the fungus. The first is the unicellular yeast form. The second is an aseptate mycelial form. The transition from the yeast form to the mycelial form is referred to as germination. Much work has gone into assessing the function of germination in infectivity.

From natural abundance C-13 NMR experiments done with the germinating yeast, my co-worker and I decided to test the applicability of the method to classification of the C. albicans. Since it is possible to serotype strains of Salmonella typhimurium, it has been assumed to be possible to do this with C. albicans. Hasenclever and Mitchell laid the groundwork for the antigenic studies of C. albicans (53). The importance of identifying the individual strains of C. albicans would be in the area of epidemiology. This information could be used to track the spread of an infection through a population, possibly identify the source of an epidemic, and be used to track the spread of strains resistant to the drugs used to treat infections. The spectra reported in the literature indicated that the natural abundance spectra were sensitive enough to distinguish between widely different organisms, e.g.- cestodes (41) and grapes (29). Our own experiences also indicated this. We gathered spectra on over twenty different strains of C. albicans, and from these spectra a pattern emerged. By assigning the resonances of the spectra to various compounds, it was possible to derive a statistical basis for the visual classification scheme that we had developed earlier that had divided strains of C. albicans into similar groups.

EXPERIMENTAL

Organisms

The yeast used in the NMR experiments were obtained from several sources. 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, NC). All strains were clinical isolates passed in culture no more than twice before being received. C. guillermondi, C. krusei, C. pseudotropicalis, C. tropicalis, and Torulopsis glabrata were from the Montana State University mycological culture collection.

Preparation and Maintenance of Organisms

Natural Abundance Experiments

For the yeast, storage was on potato flakes agar (PFA) slants at 4-6°C during the initial portion of the study and later on Sabouraud's dextrose agar slants at room temperature. Biochemical confirmation of all species was performed using API strips from Analytab Products, Ayerst Labs (Plainview, NY). Cells for NMR use were grown on

synthetic amino acid medium for fungi (SAAMF) (Table 1). The medium was prepared in accordance with the instruction provided by Gibco (Grand Island, NY) with the medium. This included the addition of 10g/l of glucose followed by filter sterilization.

For a natural abundance NMR experiment, the cells were inoculated into 2 ml of SAAMF in a 5 ml tube and incubated for 24 hours at 37°C with aeration provided by constant rotation at 160 rpm. One hundred and fifty ml of SAAMF in a 250 ml Erlenmeyer flask was inoculated with two drops of the overnight culture and incubated at 37°C, with 160 rpm for 24 hours. Cells obtained for the 28°C temperature experiments were grown in a shaking water bath rotating at 160 rpm. Cells grown on GYEP for the salt supplement experiments, followed the same protocol as the cells grown on SAAMF. Cells were collected, washed three times in 0.15 N NaCl and 0.5 - 2 x 10^{10} cells were packed into a 10 mm NMR tube. Cells were kept on ice until use.

Enriched Experiments

The yeast were maintained by the same methods as used for the natural abundance experiments. The inoculum was grown for 24 hrs. in 2 ml of GYEP at 37°C. One drop was then transferred to 10 ml of GYEP in a 25 ml Erlenmeyer. The culture was grown for 24 hrs. on a shaker at 160 rpm at 37°C. The cells were harvested, washed in sterile distilled water three times, and then kept refrigerated

Table 1. Formulation for Synthetic Amino Acid Medium Base, Fungal

Ingredients[a]	Amount (g/l)
L-arginine	1.05
L-lysine	0.58
L-histidine	0.31
L-tyrosine	0.36
L-tryptophan	0.36
L-phenylalanine	0.32
L-cysteine	0.24
L-methionine	0.15
L-threonine	0.48
L-leucine	0.52
L-isoleucine	0.52
L-valine	0.46
fumaric acid	1.50
Na-pyruvate	1.00
NH_4-acetate	0.50
$K_2HPO_4 \cdot 3H_2O$	0.50
L-glutamine	2.52
L-aspartamine	1.00
L-proline	1.00
glycine	0.50
MOPS[b]	16.45
TRIS[c]	10.45

Table 1 (continued)

Ingredients[a]	mg/l
biotin	0.50
folic acid	5.00
choline chloride	25.00
nicotinamide	5.00
di-Ca pentothenate	25.00
pyidoxal HCl	5.00
thiamine HCl	5.00
riboflavin	5.00
i-inositol	25.00
$MgCl_2$ anhydrous	95.35
$FeCl_3 \cdot 6H_2O$	2.70
$ZnSO_4 \cdot 7H_2O$	0.80
$MnSO_4 \cdot 4H2O$	0.36
$CaCl_2$ anhydrous	5.57
phenol red	2.00

[a] All chemicals came premixed in one packet from GIBCO (Grand Island, NY)

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

[c] TRIS = 2-amino-2-(hydroxymethyl)-1,3-propanediol

until use. It was found that the cells could be kept at 4°C for up to one week without affecting the results of the experiment. For the actual experiment, the cells were counted, suspended in sterile distilled water, and added to the NMR tube just before use. Final concentration in the NMR tube with the addition of the 1.5 ml of medium was 5 x 10^7 cells/ml for most experiments. The one exception used $1x10^9$ cells/ml.

The 10 mm NMR tube contained 0.50 ml of sterile 1.20 N NaCl in D_2O, 1.00 ml of sterile 1.0% yeast extract-2.0% peptone solution and 10.0 mg labeled sugar. This solution was mixed up to thirty minutes prior to the NMR run. The 10 mm NMR tube was moderately sterile in that it had been washed with a germicidal detergent and was then dried in a culture transfer hood under germicidal UV light.

NMR Parameters

All experiments were done on a Bruker WM-250 spectrometer operating in Fourier transform mode with quadrature detection. The operating frequency was 62.89 MHz. The spectrometer was interfaced to an Aspect 2000 computer. All data files were of 8k length. The natural abundance spectra were transformed using a 10 Hz line broadening factor applied by an exponential multiplication. The enriched experiments were transformed using a 5 Hz line broadening factor.

Natural abundance spectra were acquired while the temperature of the sample was maintained at 2°C ± 2° by means of the standard cooling equipment available with the Brker WM-250. The enriched spectra were acquired while the temperature was maintained at 28°C +/- 2°. Some overheating problems were found to have occurred due to the power of the broadband decoupler. This difficulty was overcome by use of a bilevel decoupling routine. The routine switched the power level from 2 watts during the acquisition of the spectrum to 0.2 watts during the delay period.

For both sets of experiments, the sweep width was from -10 to 220 ppm. The lock signal for the natural abundance experiments was provided by either a D_2O/TMS capillary, a d_6-benzene capillary or one of two external sources, a D_2O/ethanol sample or a $CDCl_3$ sample. For all of the natural abundance experiments except the saline experiments, the pulse width was 85°, the relaxation delay was 3.0 seconds and the acquisition time was 0.3 seconds. Six hundred scans were accumulated per file. For the saline experiments, the pulse width was 45° and the relaxation delay was 1.5 seconds. One thousand scans per file were accumulated. The enriched experiments used the same pulse width and delay as the saline experiments except that only 500 scans per file were accumulated. The lock signal for the enriched experiments was provided by the D_2O

in the 0.5 ml of 1.200 N NaCl solution that was added to the medium in the NMR tube.

Oxygen was provided by a pair of capillaries (Fig. 1). The design of this system was suggested by the coaxial system developed by den Hollander for use in his experiments (54). Two 2mm diameter glass tubes were used. The longer, finer capillary was coaxial and went to the bottom of the 10mm tube. Its function was to provide mixing and oxygen in such a way as to minimize field perturbations. This tube had a bubbling rate of approximately 2 ml/min. The larger diameter tube was not coaxial, but was above the sensing coils of the NMR. It provided a high rate of oxygen bubbling (20 ml/min) with larger bubbles. The two tubes were held in place by a modified Teflon vortex plug.

Due to loss of fluid during the experiments, it became necessary to bubble the oxygen through an Erlenmeyer flask half filled with heated water (40°C).

Identification

Natural Abundance Experiments

Identification of the compounds was accomplished by comparison of the resonances in the experimental spectra to spectra of known compounds.

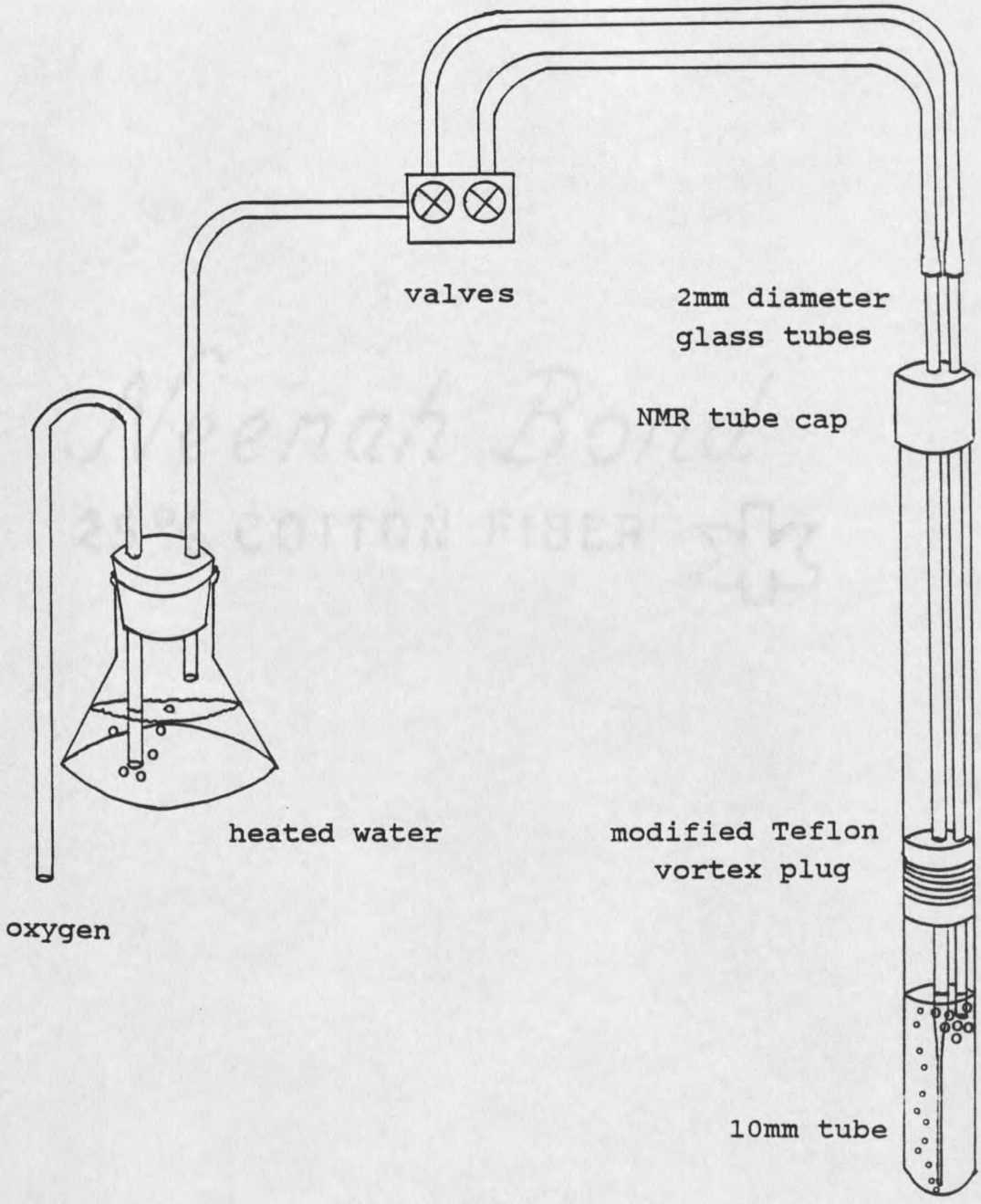

Figure 1. Apparatus used to bubble oxygen into the 10 mm NMR tube during the data acquisition.

Enriched Experiments

At the point during an enriched experiment when the glucose was nearly depleted, the NMR tube was removed from the spectrometer and put on ice. The cells were then immediately separated from the supernatant liquid by thrice repeated centrifugation and washing. The supernatant liquid and washings were combined and filtered through 0.45 μm Millipore filters. One sample was freeze-dried to reconcentrate it. Methanol was added as an internal reference and then analyzed on the NMR using the same parameters as those used during an enriched experiment except that the temperature was uncontrolled but remained near 22°C. The resulting spectra were compared to the resonances obtained from external standards.

The cells were harvested by centrifugation and the cell walls were fractured by use of the glass bead technique (55). The fractured cells and their contents were filtered on 0.45 μm Millipore filters and the filtrate was freeze-dried and then analyzed using the same protocols as the supernatant liquid samples.

RESULTS

Natural Abundance Carbon-13 Studies of Candida albicans

Figure 2 is a spectrum typical of those obtained by natural abundance C-13 NMR. The example is a spectrum of the yeast Candida albicans showing the transformed spectrum from 0-200 ppm. The line widths of the peaks ranged from 10-15 Hz.

The assignments of the resonances of this and other spectra of the yeasts were confirmed by comparison to external standards. The standards were run under conditions that as closely as possible mimicked those of the experiment. The identifications were cross-checked by comparison to reports of high concentration compounds in yeast and particularly C. albicans. Sullivan et al extracted cells of C. albicans and identified some of the contents by the standard methods of chromatography (56).

The region from 0-55 ppm is dominated by the resonances from free amino acids. The largest peaks are due to the methylene resonances of glutamic acid at 27.2, and 33.5 ppm and the α-carbon at 54.9 ppm. Often associated with these peaks are the analogous resonances of glutamine at 26.7,

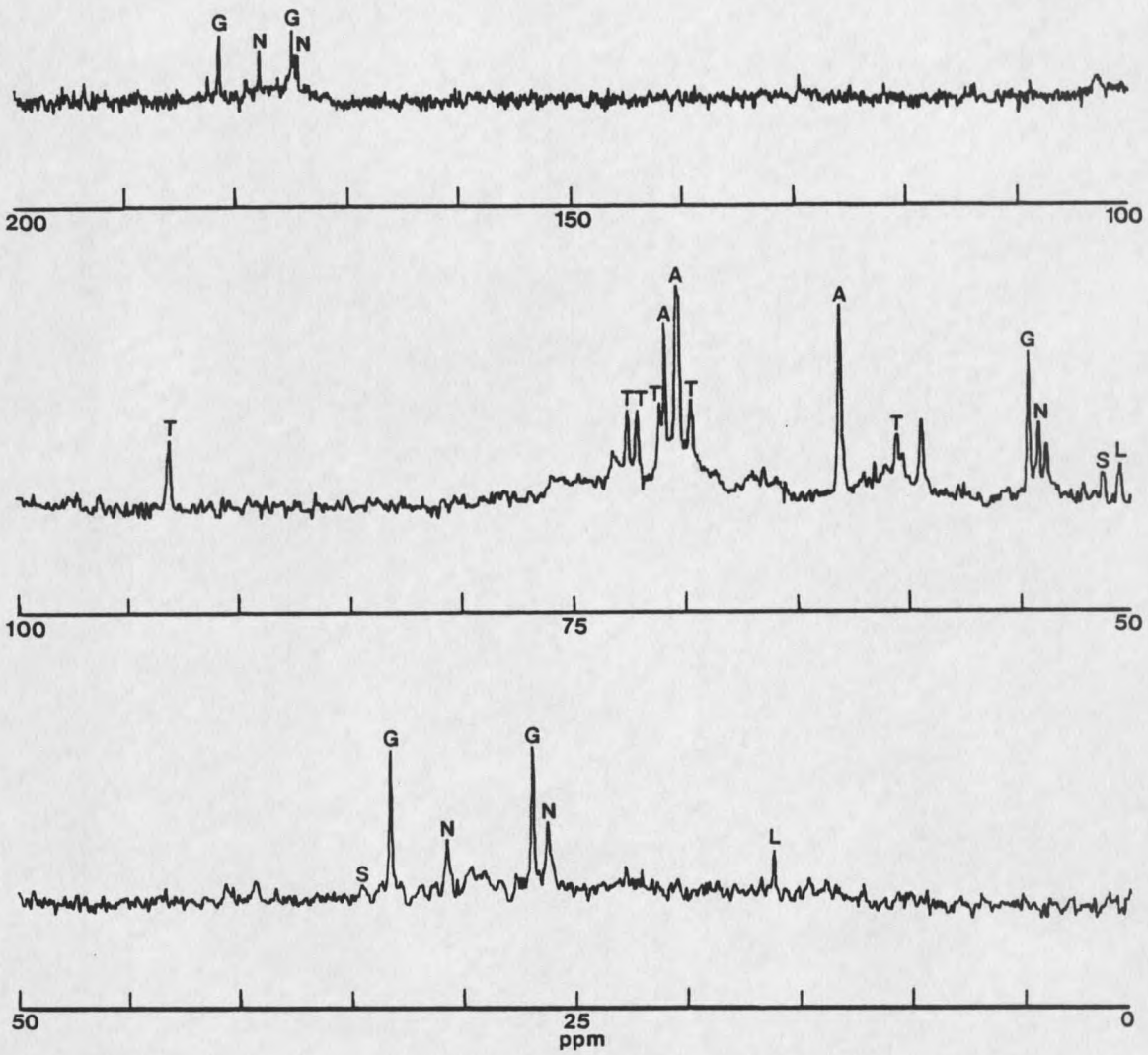

Figure 2. C-13 NMR spectra of C. albicans strain 118 grown 24 hours on SAAMF. The abbreviations are: A arabinitol, G glutamate, N glutamine, Y glycerol, L alanine, T trehalose, R aspartate, P proline and S aspartamine.

31.1, and 54.4 ppm. Alanine, another amino acid that is frequently seen, resonates at 16.4 and 50.9 ppm.

Occasionally visible in other spectra are proline at 24.1, 29.4, 46.3 and 61.3 ppm and ethanol at 17.1 and 57.8 ppm.

In the region from 55-110 ppm, the major resonances are due to the alcohol groups on carbohydrates, their hemiacetals and hemiketals, and the amino carbons of some compounds. One of the major carbon compounds found in C. albicans is arabinitol. It dominates this region of the spectrum with its resonances at 63.3, 63.4, 70.6, 70.8 and 71.3 ppm. Typically the resonances for the C-1 and C-5 carbons at 63.4 and 63.3 overlap to such an extent that they cannot be resolved into separate peaks. This is often true of the C-2 and C-3 resonances at 70.6 and 70.8 ppm. Although the intensities of the individually resolved resonances should be equivalent, overlap with neighboring resonances and the use of an exponential line broadening technique can alter the relative heights. Another polyol detected in Candida albicans was glycerol, with peaks at 62.8 ppm for the C-1 and C-3 carbons, 72.4 ppm for the C-2 carbon. Ribitol, another five carbon polyol, also has resonances at 62.7, 72.3 and 73.6 ppm. Glycerol was chosen as the compound responsible for the resonances seen in the spectra because of the relative intensities of the peaks. The glycerol 62.8 peak would have an intensity

approximately twice that of the 72.4 peak. Ribitol would have nearly equal intensities for all peaks. The other major carbohydrate component in the cells is trehalose. Trehalose is an α-1,1-diglucoside. It has resonances at 93.3, 72.8, 72.4, 71.3, 69.9 and 60.7 ppm. Not all of the resonances were visible in all spectra due to overlap with neighboring resonances.

There is also a resonance at 59.4 ppm. In some spectra, this resonance is almost four times the height of any other peak in the spectra. No reasonable assignment could be made for this resonance.

Resonances in the area of 110-140 ppm are due to the aromatic amino acids and to the unsaturated fatty acids (44). These peaks are usually not very well defined and are often below the noise level. The region out to 200 ppm is dominated by the carboxylate resonances from the amino acids and esters of the fatty acids. These occur from 165-180 ppm.

Most of the compounds of interest and most of the changes in the spectra associated with these compounds occur in the region from 0 to 100 ppm. For this reason, all of the following spectra will span only this region.

C. albicans was studied in more detail by analyzing a total of 23 strains using C-13 NMR. For the baseline data, the growth parameters were kept the same for all of the strains. The cells were grown on SAAMF for 24 hours at

37°C. SAAMF was chosen because it is a chemically-defined medium and should not have any batch to batch variations.

Under the same conditions of growth, the strains of C. albicans can be divided into three groupings, type I, type II and type III, based upon their natural abundance spectra (Fig. 3). The type I classification encompassed the largest number of strains, seventeen. Strain 118 was arbitrarily chosen as the archetypical strain. The type II grouping was the second largest with 5 organisms. Strain 122 was chosen as the typical organism of this group. The type III group was represented solely by strain 117, and was formed by default since strain 117 fit neither the type I nor the type II spectral patterns.

Type I organisms distinguish themselves by the abundance of peaks their NMR spectra reveal, and the relative equality in the intensity of those peaks (Fig. 4). As previously mentioned, glutamate, glutamine, and alanine make up the major resonances in the region from 0-55 ppm. Arabinitol and trehalose dominate the carbohydrate region.

The type II strains are also unique in that the arabinitol peaks at 63.3, 70.4 and 70.9 ppm dominate the entire spectrum (Fig. 5). The higher concentration of arabinitol is the cause of the apparent higher signal to noise of type II spectra compared to those of other types. The concentration of glycerol is very low in type II cells. Trehalose is also present, but it also has a low

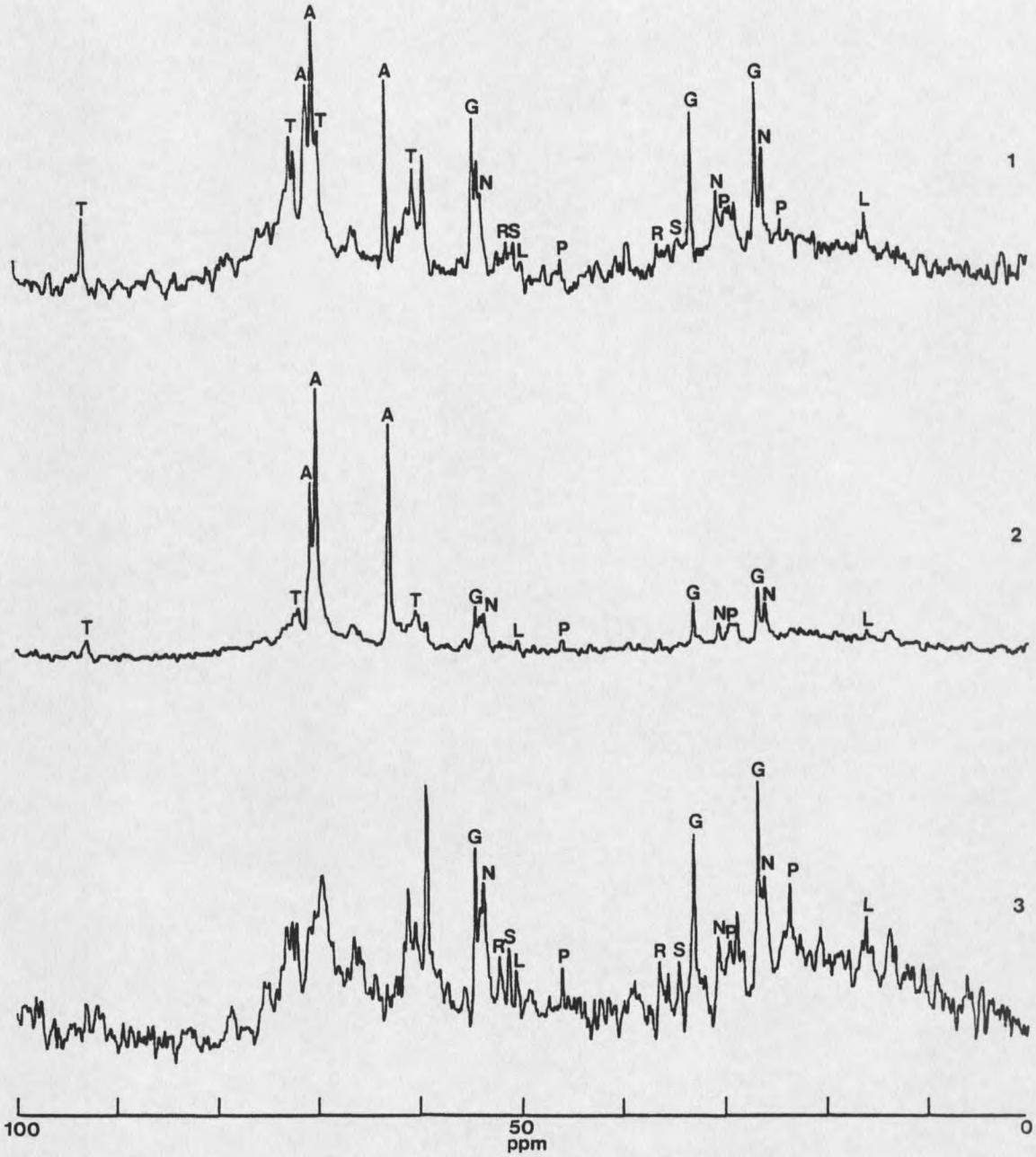

Figure 3. C-13 NMR spectra of C. albicans strains 118 (type I), 122 (type II) and 117 (type III) grown 24 hours on SAAMF. The abbreviations are the same as used in Figure 2.

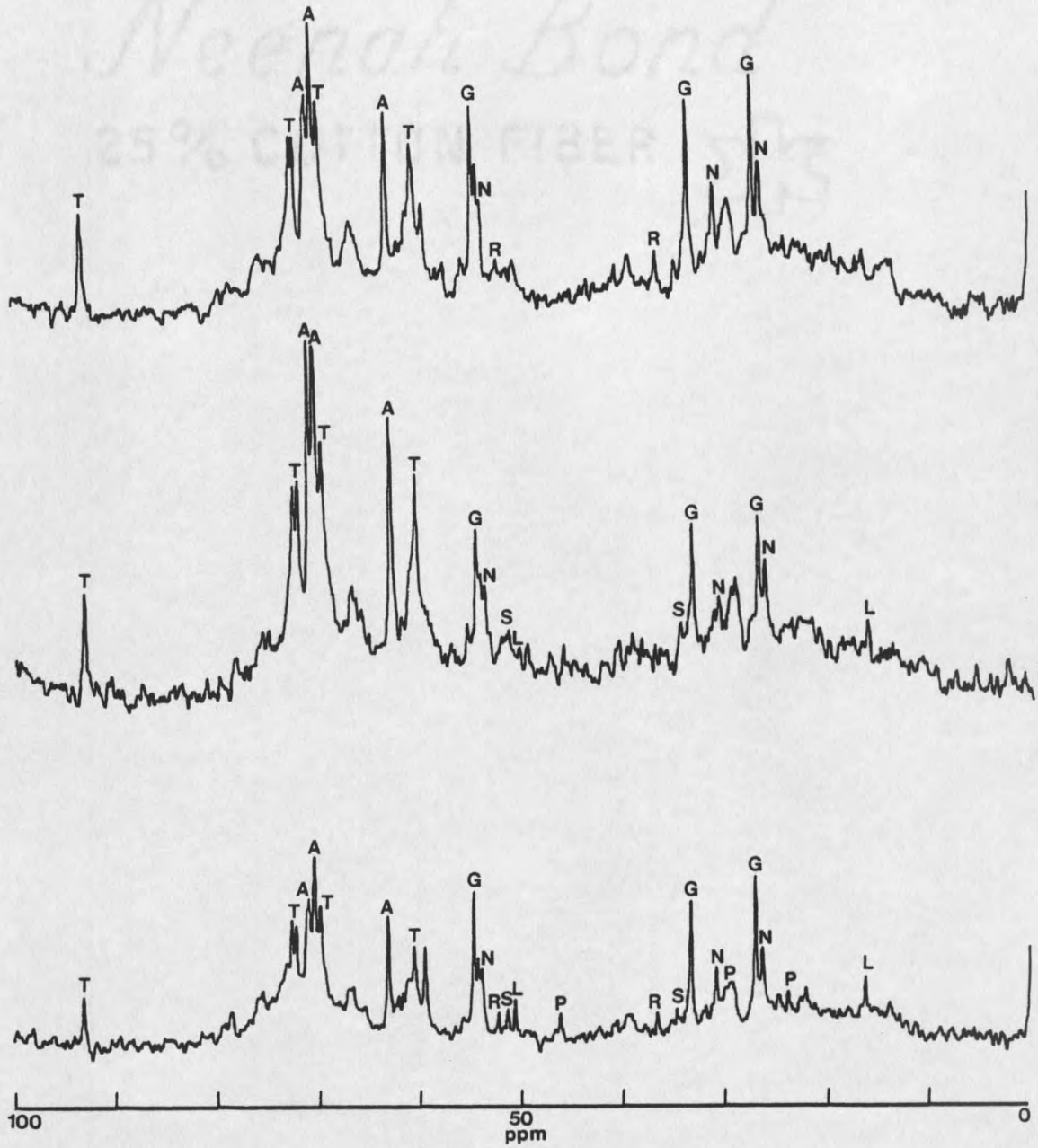

Figure 4. C-13 NMR spectra of three type I strains of C. albicans, 1) strain 104, 2) strain 130 and 3) strain 163. The abbreviations are the same as used in Figure 2.

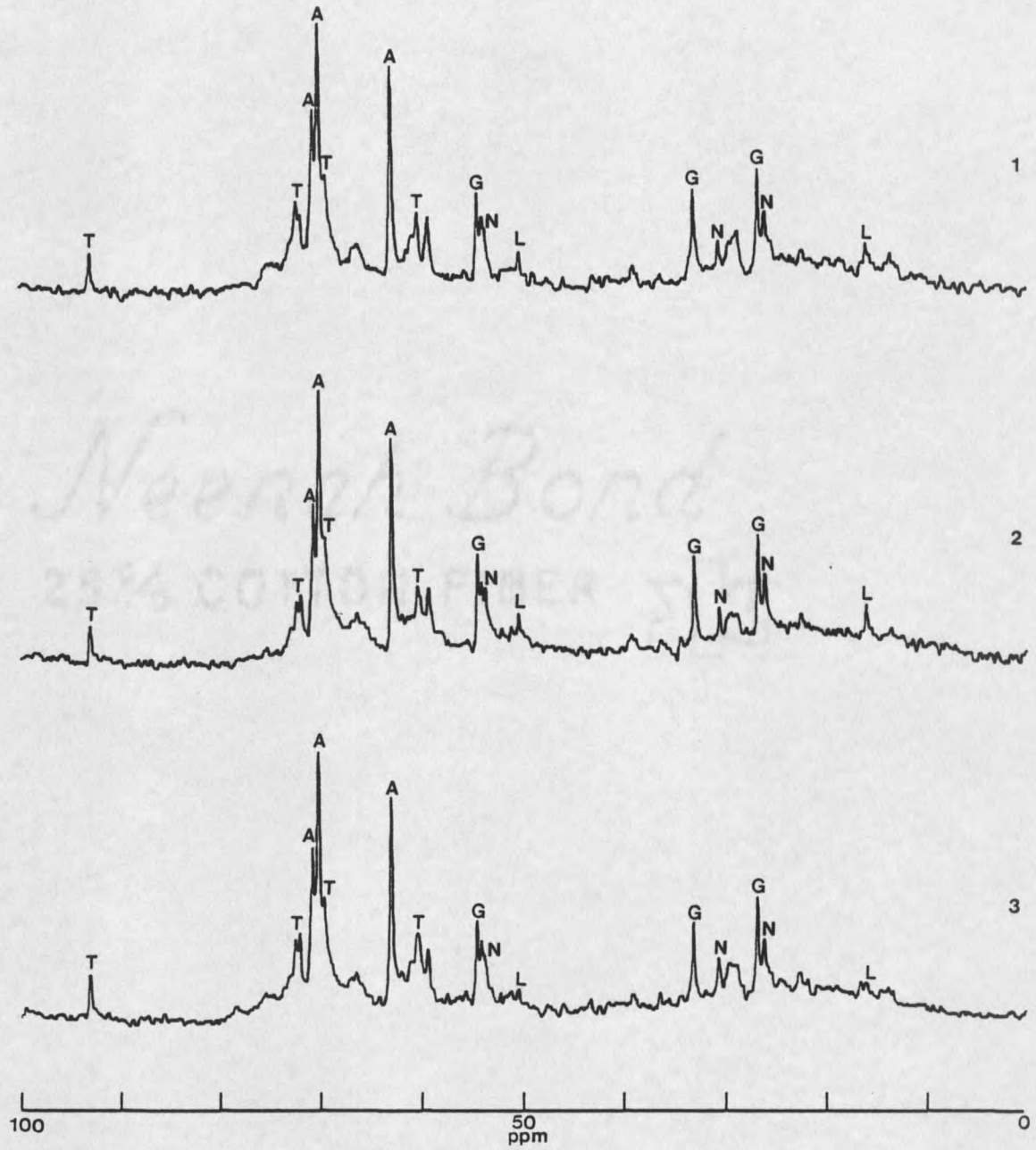

Figure 5. C-13 NMR spectra of three type II strains of C. albicans, 1) strain 147, 2) strain 135 and 3) strain 113. The abbreviations are the same as used in Figure 2.

concentration in type II cells. Its presence is often marked by the appearance of the peak at 93.1 ppm. There are still the amino acid resonances of glutamate at 27.2, 33.5, and 54.9 ppm, glutamine at 26.7, 31.1, and 54.4 ppm, and alanine at 16.4 and 50.6 ppm, but their intensities are rather small compared to the arabinitol peaks.

The sole type III strain has a spectrum that is notable for its absence of arabinitol (Fig. 3). Conspicuous in this organism are the intensities of the resonances at 24.1, 29.4, 46.3 and 61.2 ppm. These are indicative of a high level of proline in the organism. The presence of glutamate is indicated by the peaks at 27.2, 33.5, and 54.9 ppm, as is glutamine by its analogous peaks at 26.7, 31.1 and 54.4 ppm. Unlike either of the other two types, the dominant resonance is at 59.6 ppm. The carbohydrate region is also of note because of the apparent lack of the resonances associated with trehalose. The major peaks that do occupy the carbohydrate region of the spectrum could not be assigned.

The levels of arabinitol were correlated to the concentration of NaCl in the solution for type I strains, type II strains and type III strains. With no added saline, the cells show the typically complex spectrum associated with growth on GYEP. Trehalose and arabinitol are present at a concentration typical of a type I strain (Fig. 6). The glutamate levels are higher than is often

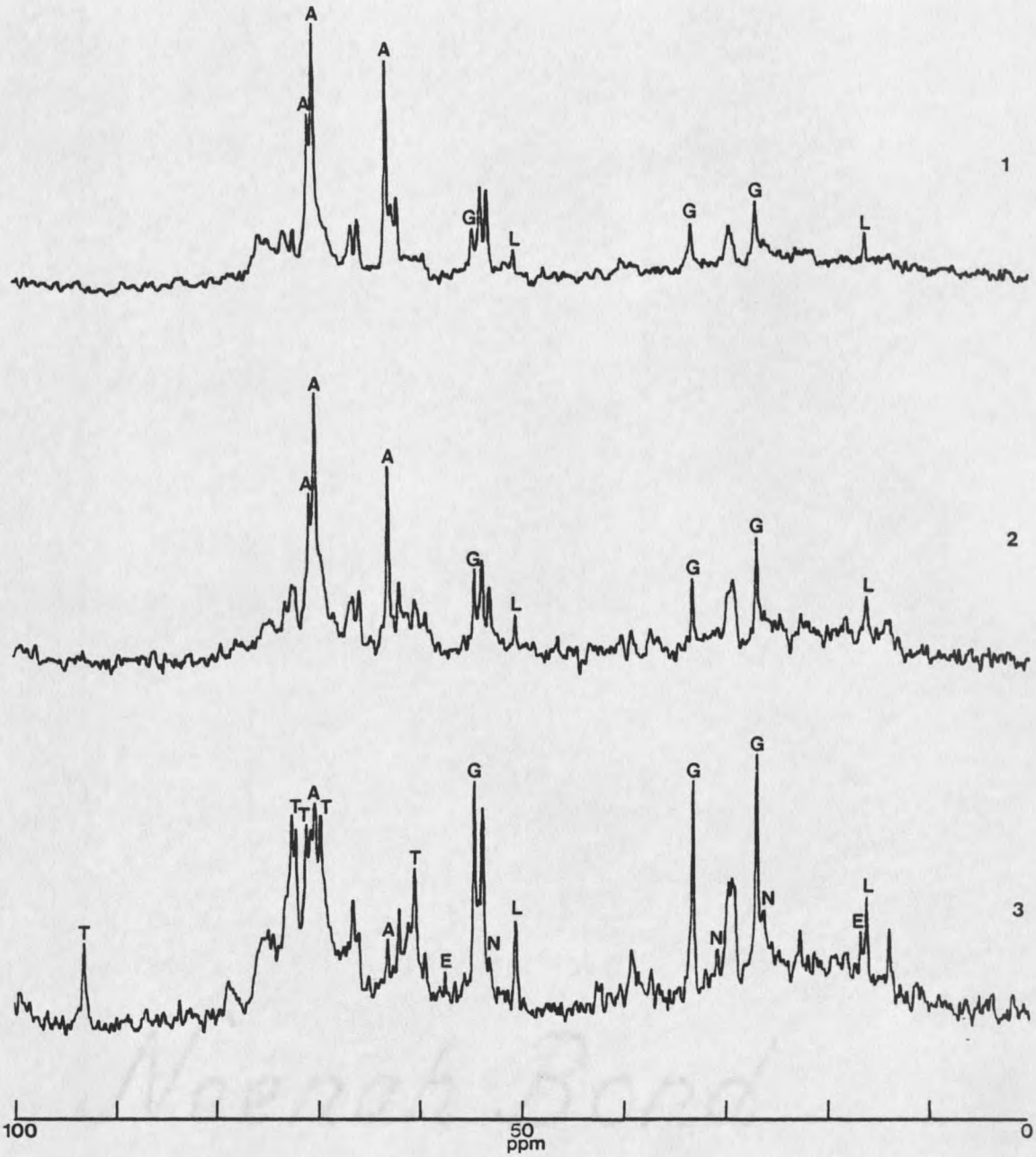

Figure 6. C-13 NMR spectra of C. albicans type I grown on GYEP supplemented with various concentrations of NaCl, 1) 0.150 N NaCl, 2) 0.075 N NaCl and 3) no added NaCl. The abbreviations are the same as used in Figure 2.

encountered, but within acceptable margins. Alanine is also present. With the concentration as low as 0.075 N NaCl, the spectrum has drastically changed (Fig. 6). The trehalose peaks are absent and the arabinitol resonances dominate the spectrum. As the saline concentration increases, the arabinitol stores of the cells steadily increase, until another change takes place when the concentration of NaCl reaches 0.300 N (Fig. 7). At this concentration of salt, the resonances of glycerol begin to appear in the spectrum. When the concentration of saline is 0.600 N, the spectrum is dominated by the peaks due to glycerol. Arabinitol still makes a major contribution to the spectrum, but glycerol is now the major polyol stored by the cells.

The type II and type III strains follow this same pattern, with some differences (Figs. 8-11). The type II cells had the highest final concentration of glycerol of any of the three strains tested. The type III cells were unusual in that the spectra of the cells at 0.075 N saline had a lower S/N than the other two spectra. The type III cells grown in the 0.300 N and 0.600 N saline media had a broad resonance centered at 75 ppm that was not present in the spectra of the other types. The absence of the amino acid resonances 20 ppm to 35 ppm is a singular feature of the type III cells grown in 0.600 N NaCl supplemented GYEP.

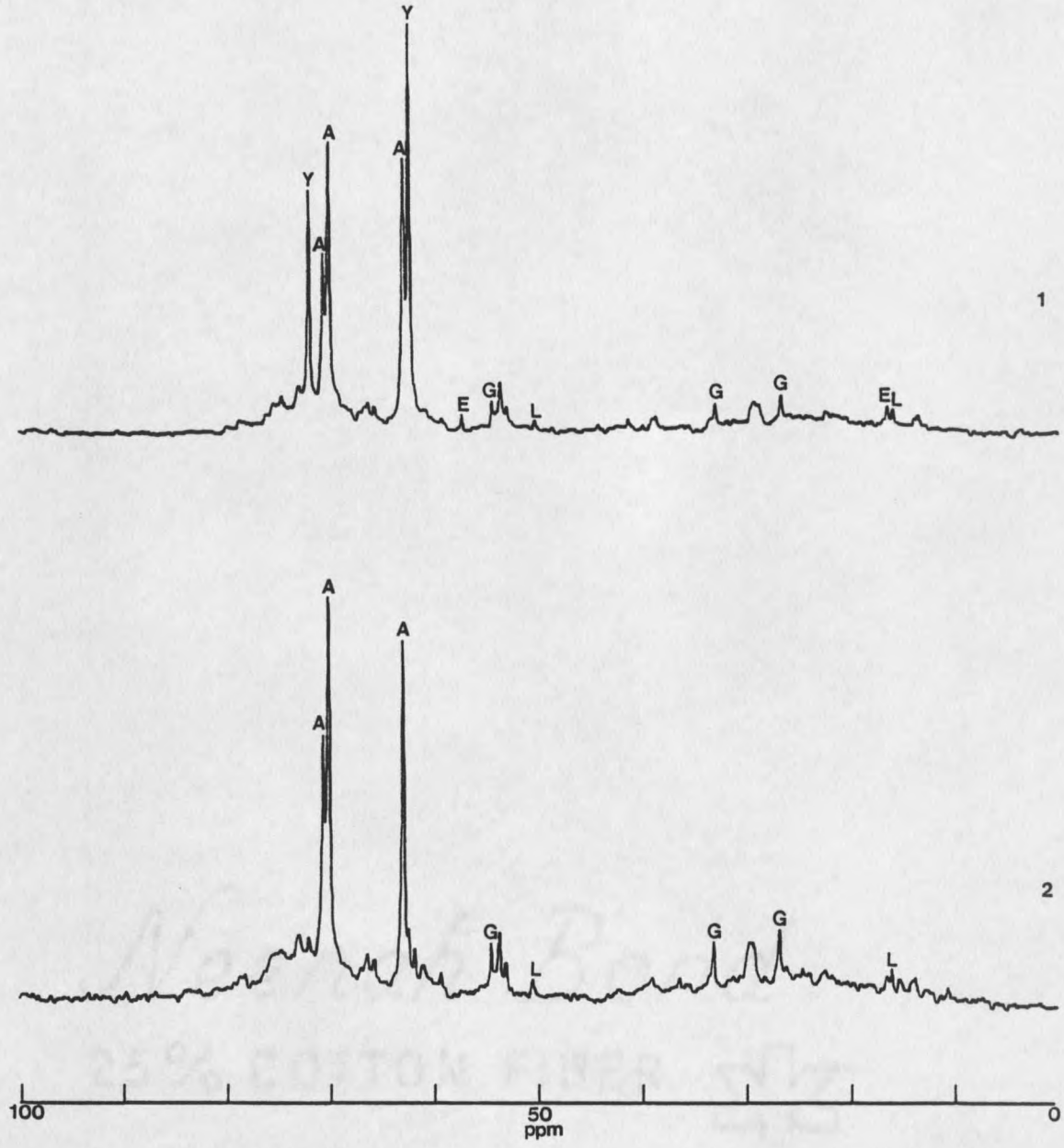

Figure 7. C-13 NMR spectra of C. albicans type I grown on GYEP supplemented with various concentrations of NaCl, 1) 0.600 N NaCl and 2) 0.300 N NaCl. The abbreviations are the same as used in Figure 2.

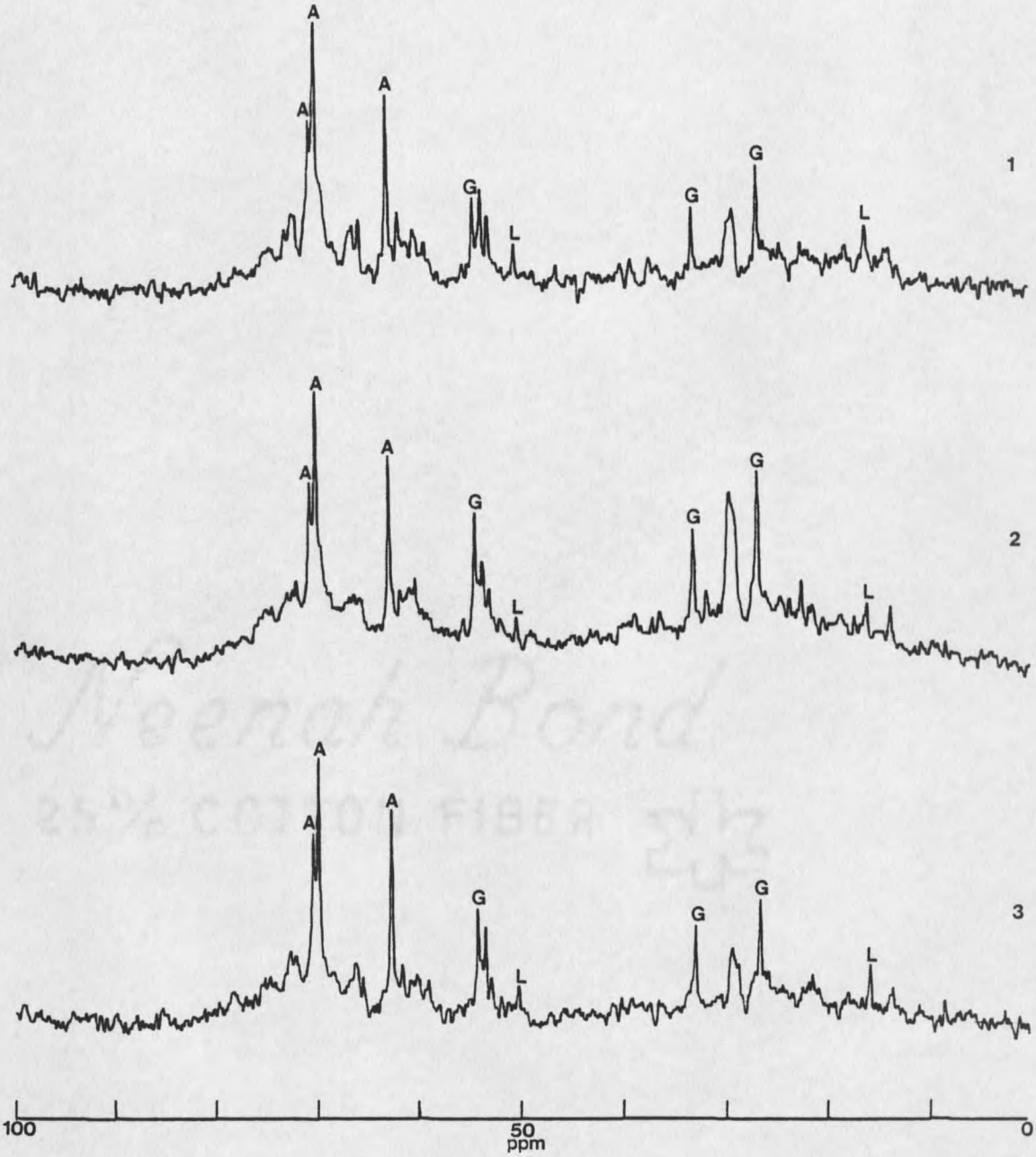

Figure 8. C-13 NMR spectra of C. albicans type II grown on GYEP supplemented with various concentrations of NaCl, 1) 0.150 N NaCl, 2) 0.075 N NaCl and 3) no added NaCl. The abbreviations are the same as used in Figure 2.

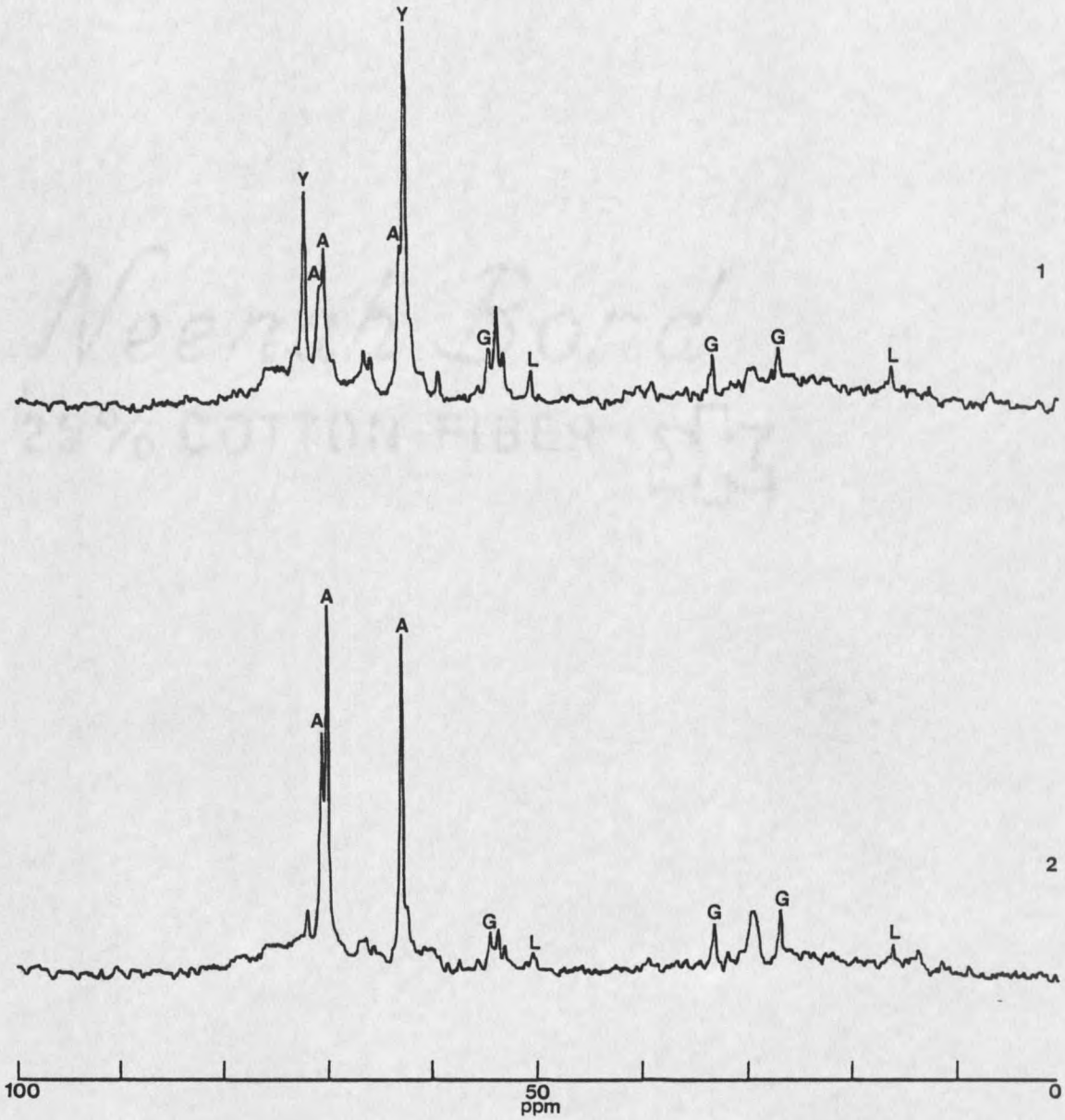

Figure 9. C-13 NMR spectra of C. albicans type II grown on GYEP supplemented with various concentrations of NaCl, 1) 0.600 N NaCl and 2) 0.300 N NaCl. The abbreviations are the same as used in Figure 2.

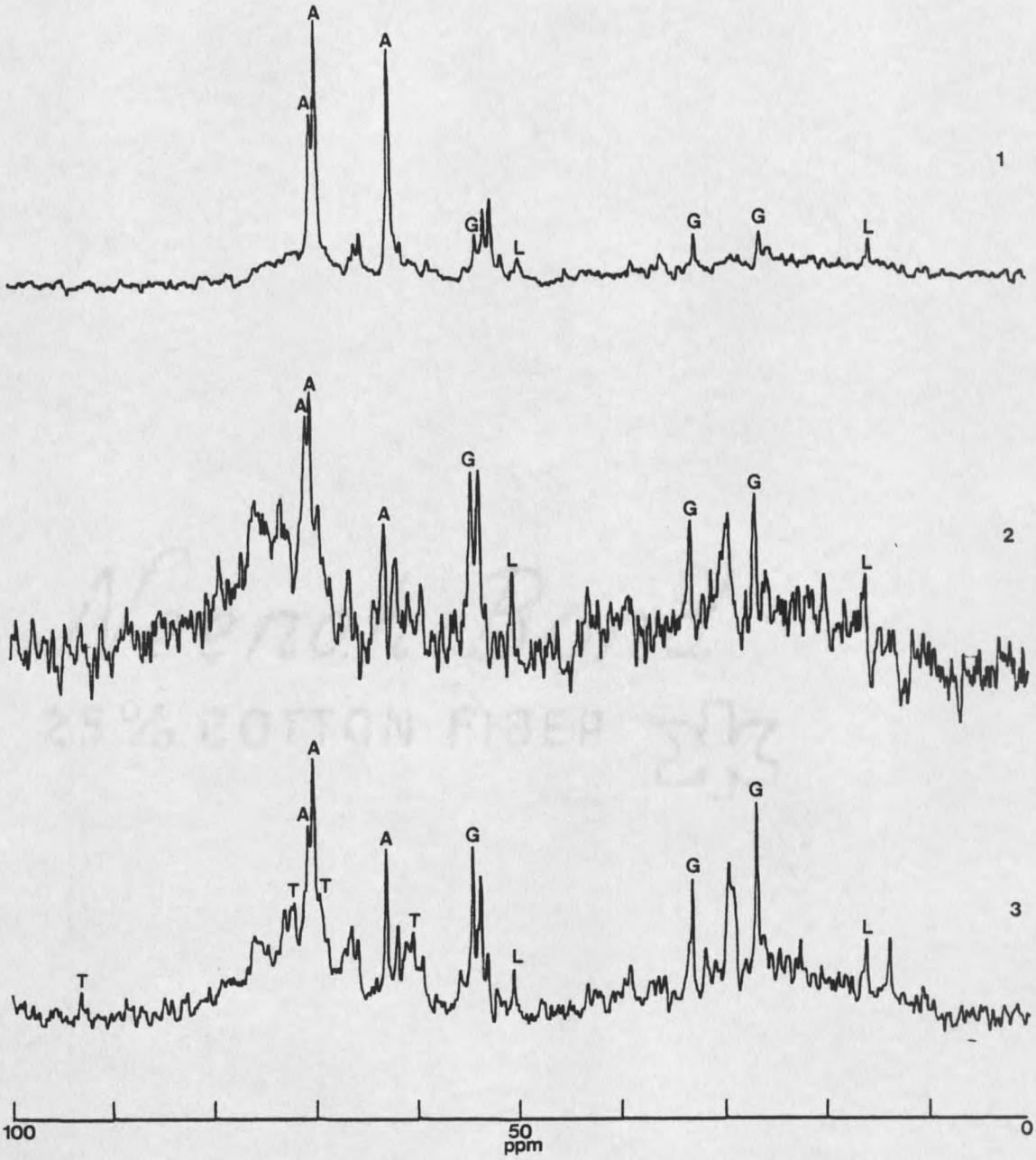

Figure 10. C-13 NMR spectra of C. albicans type III grown on GYEP supplemented with various concentrations of NaCl, 1) 0.150 N NaCl, 2) 0.075 N NaCl and 3) no added NaCl. The abbreviations are the same as used in Figure 2.

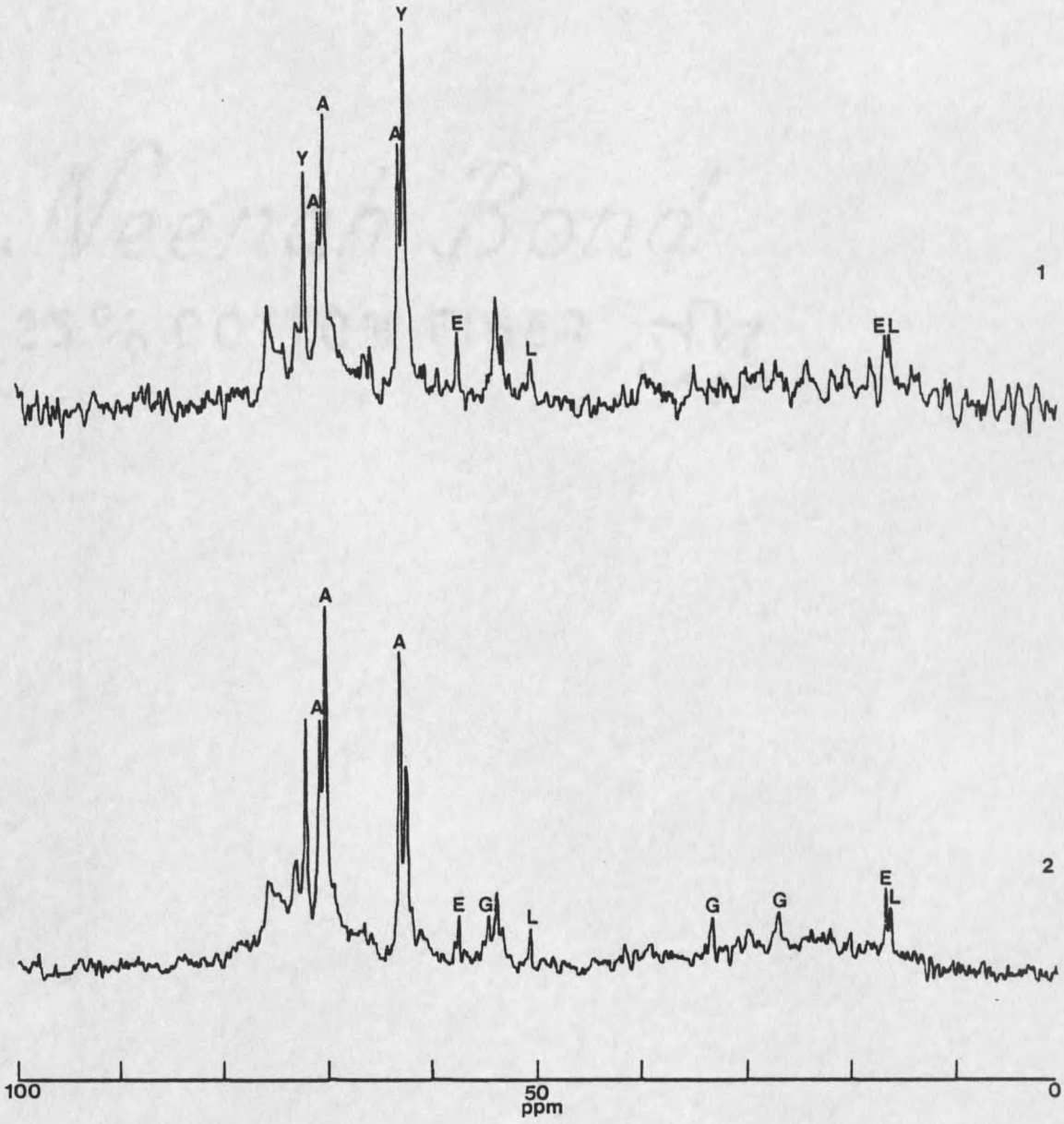

Figure 11. C-13 NMR spectra of C. albicans type III grown on GYEP supplemented with various concentrations of NaCl, 1) 0.600 N NaCl and 2) 0.300 N NaCl. The abbreviations are the same as used in Figure 2.

The temperature experiments consisted of growing type I and type II cells at two different temperatures, 28°C and 37°C using SAAMF as the growth medium (Fig. 12). These two temperatures were chosen for study because they are used extensively for inducing germination in C. albicans. The results show a dramatic change in the physiology of the type I organism. At the cooler temperature, the cells accumulated sufficient arabinitol and had low enough levels of the other carbohydrates to be classified as a type II strain. The ratio of the α-methylene peak of glutamic acid at 33.5 ppm to the C-2/C-3 peak of arabinitol at 70.2 ppm decreased in both organisms as a result of the temperature difference. At 37°C, the ratio was 0.80 for the type I strain and 0.29 for the type II strain. This changed to 0.34 and 0.14, respectively at 28°C. Also of note was the increase in the intensity of the peaks at 62.3 and 72.4 ppm in the 28°C type II spectrum.

Labeled NMR Studies

The arabinitol metabolism of C. albicans was investigated using 2-C-13-glucose, 1-C-13-glucose and 1-C-13-xylose. The results shall be discussed in that order.

2-C-13-Glucose Experiments

Figure 13 shows a typical result of an experiment that used 2-C-13-glucose as the carbon source. The spectra cover the range from 45 ppm to 73.5 ppm. This range was

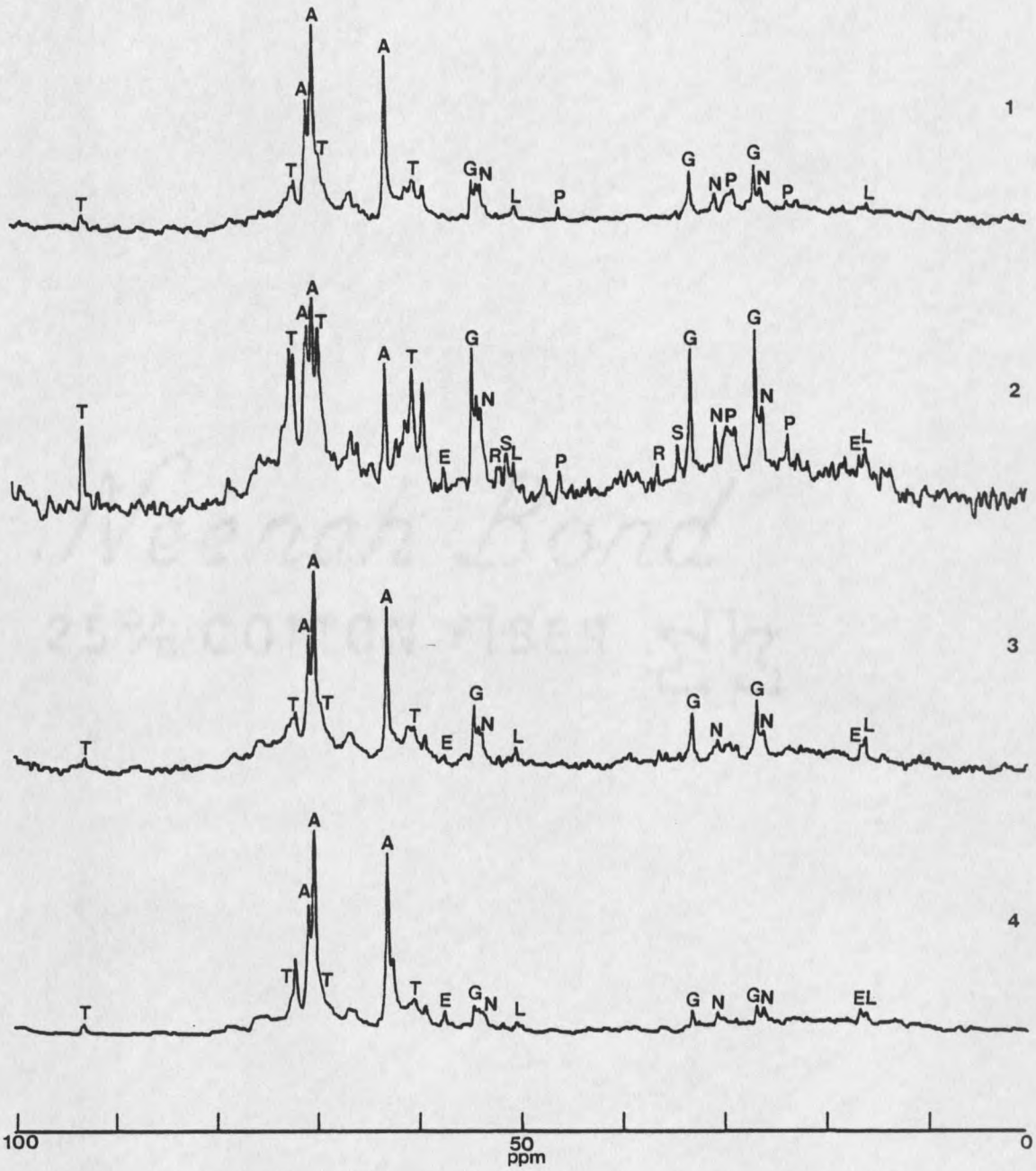

Figure 12. C-13 NMR spectra of C. albicans type I and type II grown at different temperatures, 1) type II at 37°C, 2) type I at 37°C, 3) type I at 28°C and 4) type II at 28°C. The abbreviations are the same as used in Figure 2.

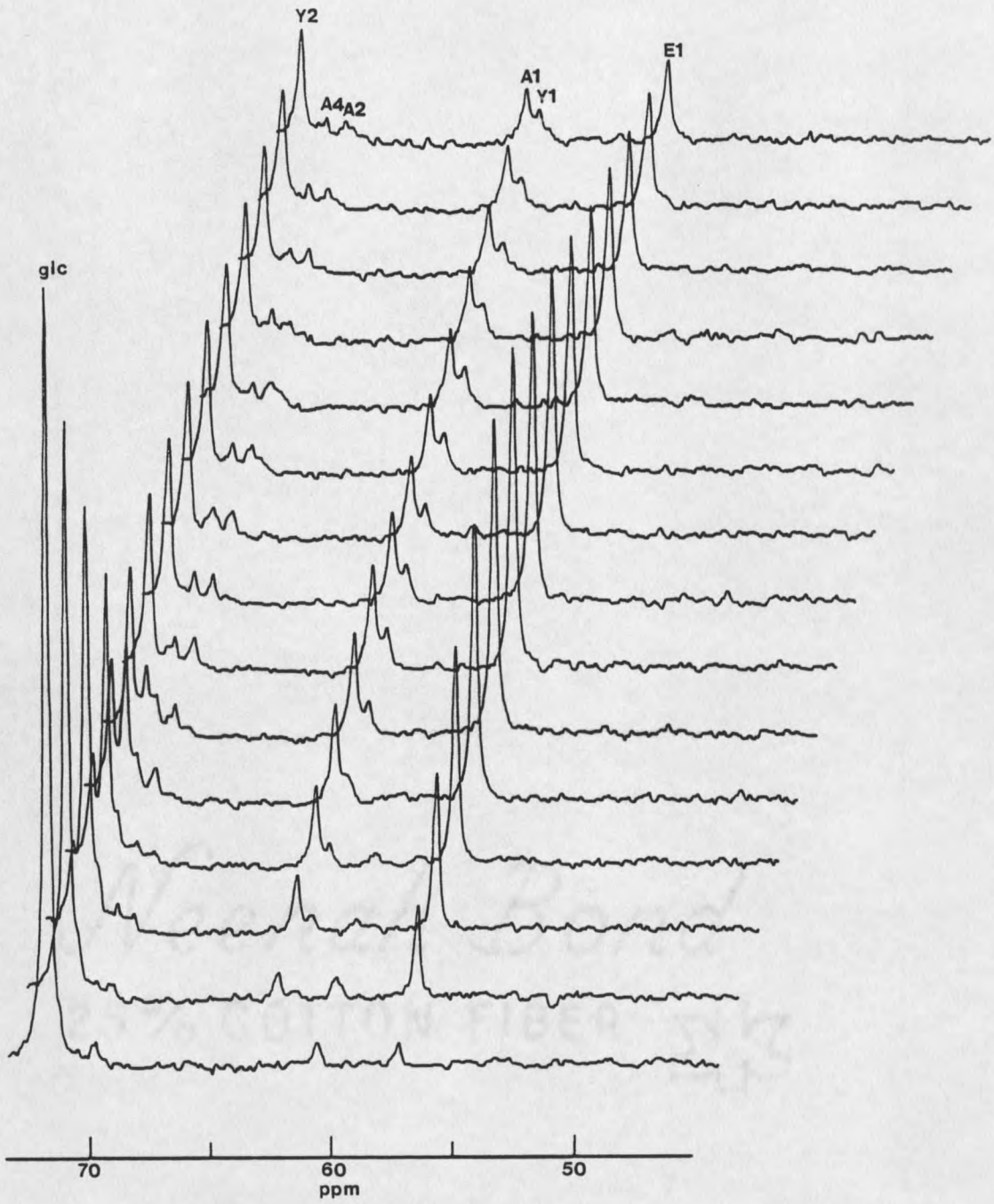

Figure 13. C-13 NMR spectra of the results of C. albicans strain 118 acting upon 2-C-13-glucose. Each spectrum represents a 15 minute accumulation. The abbreviations are: A arabinitol, E ethanol, Y glycerol, glc glucose and xyl xylose.

chosen so as to minimize the overlap of the peaks and to permit observation of all the relevant peaks. The resonance at 71.9 ppm was due to the enriched carbon of the labeled glucose as split by the anomeric carbon. The resonances at 72.4 and 62.8 ppm represent two-labeled and one-labeled and one, three-labeled glycerol, respectively. The peaks for the one and three positions on glycerol have identical chemical shifts. The resonances 70.6, 71.3 and 63.4 ppm were due to the four-, two- and one-labeled arabinitol, respectively. The resonance at 57.8 ppm was due to the production of one-labeled ethanol. A resonance at 17.1 ppm was detected and attributed to 2-C-13-ethanol. It was not included in the spectra shown for reasons of scale and because its presence does not change the analysis of the results. As expected, the spectra show metabolite production continuing until the glucose resonance is gone, at which point consumption of the polyols becomes visible. Ethanol evaporated slowly under the conditions of the experiment, accounting for the decrease in that resonance. The results of one of the 2-C-13-glucose experiments done with type I C. albicans (Fig. 13) were graphed (Fig. 14). As would be expected, ethanol had the most rapid production and the highest concentration of any of the end products. The second greatest rate and final concentration was due to 2-C-13-glycerol. 1-C-13-arabinitol had the third fastest production rate, while the rates associated with the other

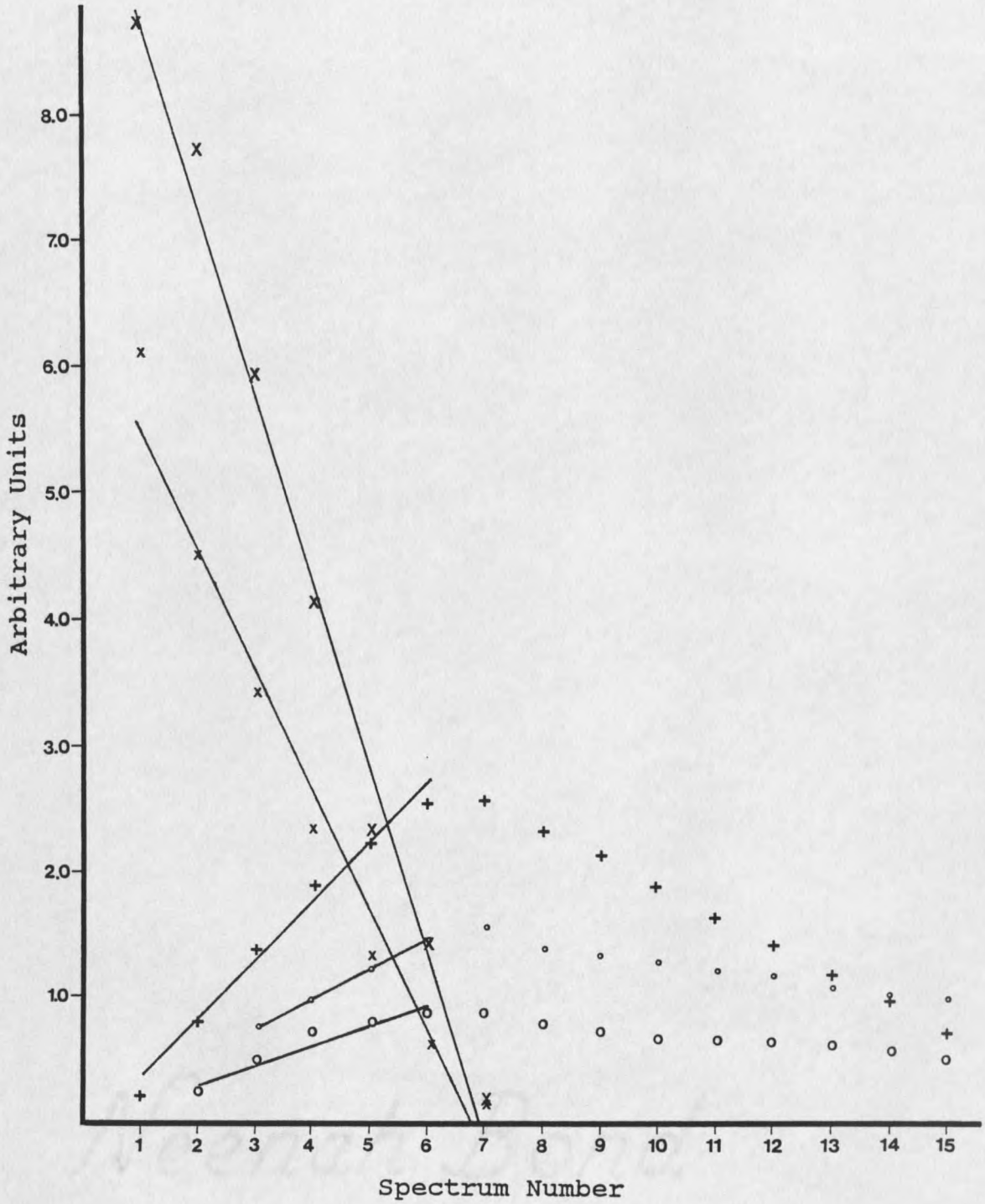

Figure 14. Graph of the results of Figure 13. The symbols used are: x β-glucose, x α-glucose, + 2-C-13-ethanol, ∘ 2-C-13-glycerol, o 2-C-13-arabinitol.

labeled arabinitols could not be measured in the spectra due to overlap with the glucose peaks. Figures 15 and 16 show duplications of the experiment shown in Figure 13. The experiment shown in Figure 17 was similar to the experiments shown in Figures 13, 15 and 16. Figure 17 was included because it covers the region from 5-76 ppm. As the 2-C-13-ethanol resonance was the only peak visible in that region of the spectra and that peak was never very intense, the other figures that show experiments that used 2-C-13-glucose as the substrate do not include the region from 0-55 ppm. The metabolism of the type II and type III organisms, strain 122 and 117, respectively, were also examined. No significant variations where found (Figs. 18, 19). Figure 20 shows the results of feeding the two-labeled glucose to a type I yeast culture containing 10^9 cells/ml. Although the cell density was almost two orders of magnitude greater than that used in the other experiments, no new peaks were visible.

1-C-13-Glucose Experiments

The 1-C-13-glucose experiments reveal further label scrambling (Fig. 21). As the spectra show, one-labeled arabinitol, one-labeled glycerol and two-labeled ethanol accumulate. There is also an unassigned peak present at 93.6 ppm. The results are analogous to those of the two-labeled glucose experiments since ethanol is the most abundant product and the concentration of glycerol is

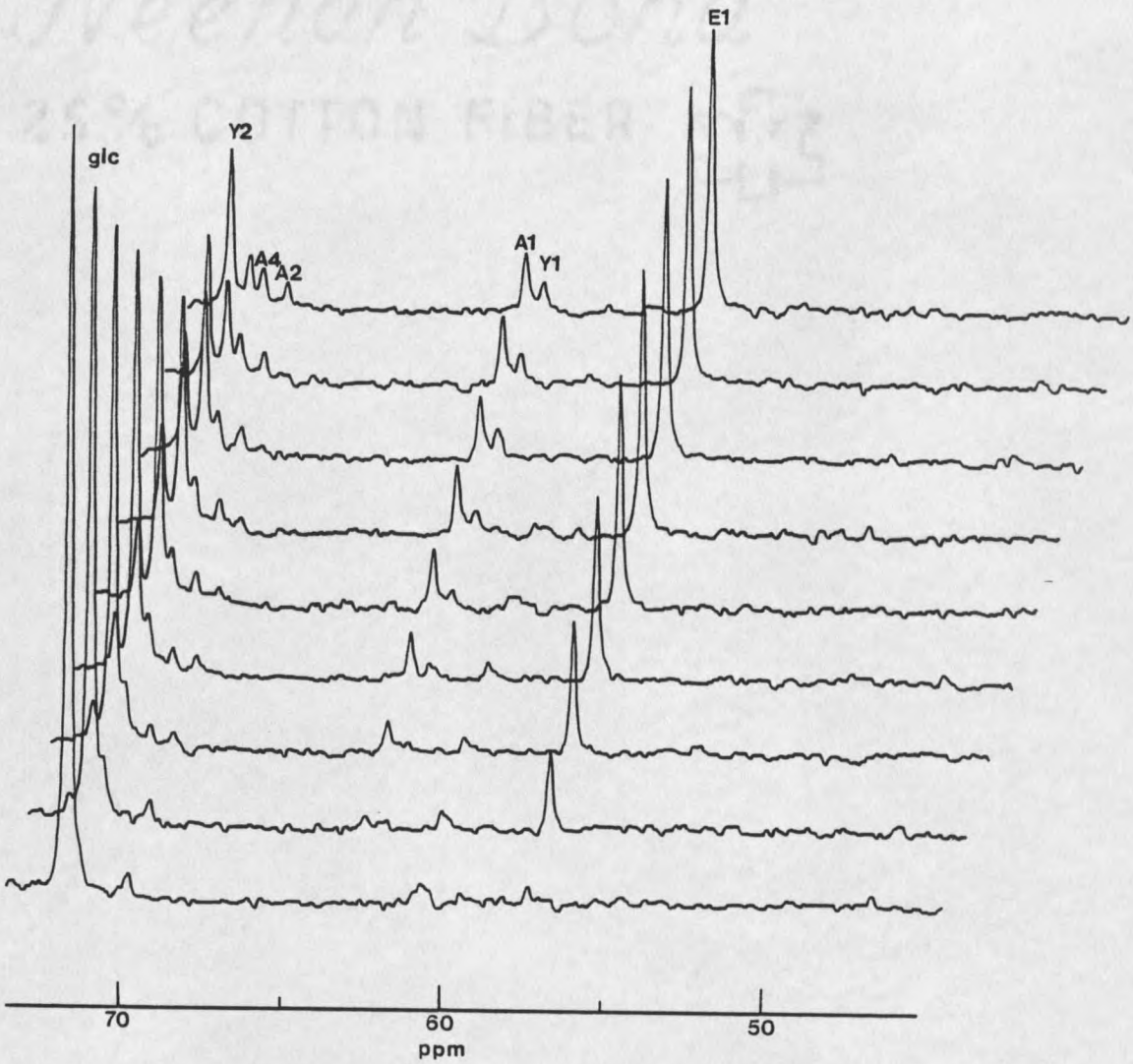

Figure 15. C-13 NMR spectra of the results of C. albicans strain 118 acting upon 2-C-13-glucose. Each spectrum represents a 15 minute accumulation. The abbreviations are the same as those used in Figure 13.

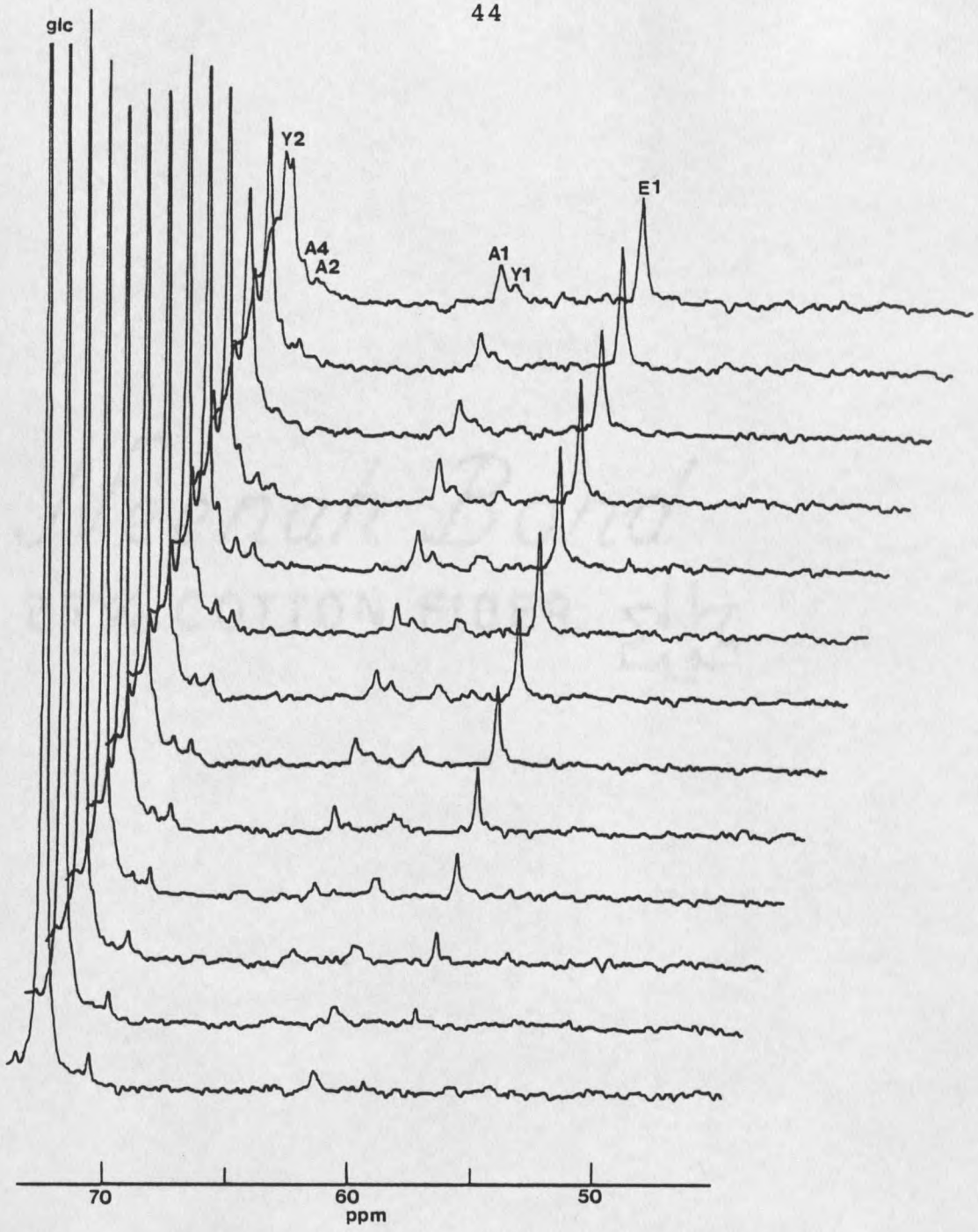

Figure 16. C-13 NMR spectra of the results of C. albicans strain 118 acting upon 2-C-13-glucose. Each spectrum represents a 15 minute accumulation. The abbreviations are the same as those used in Figure 13.

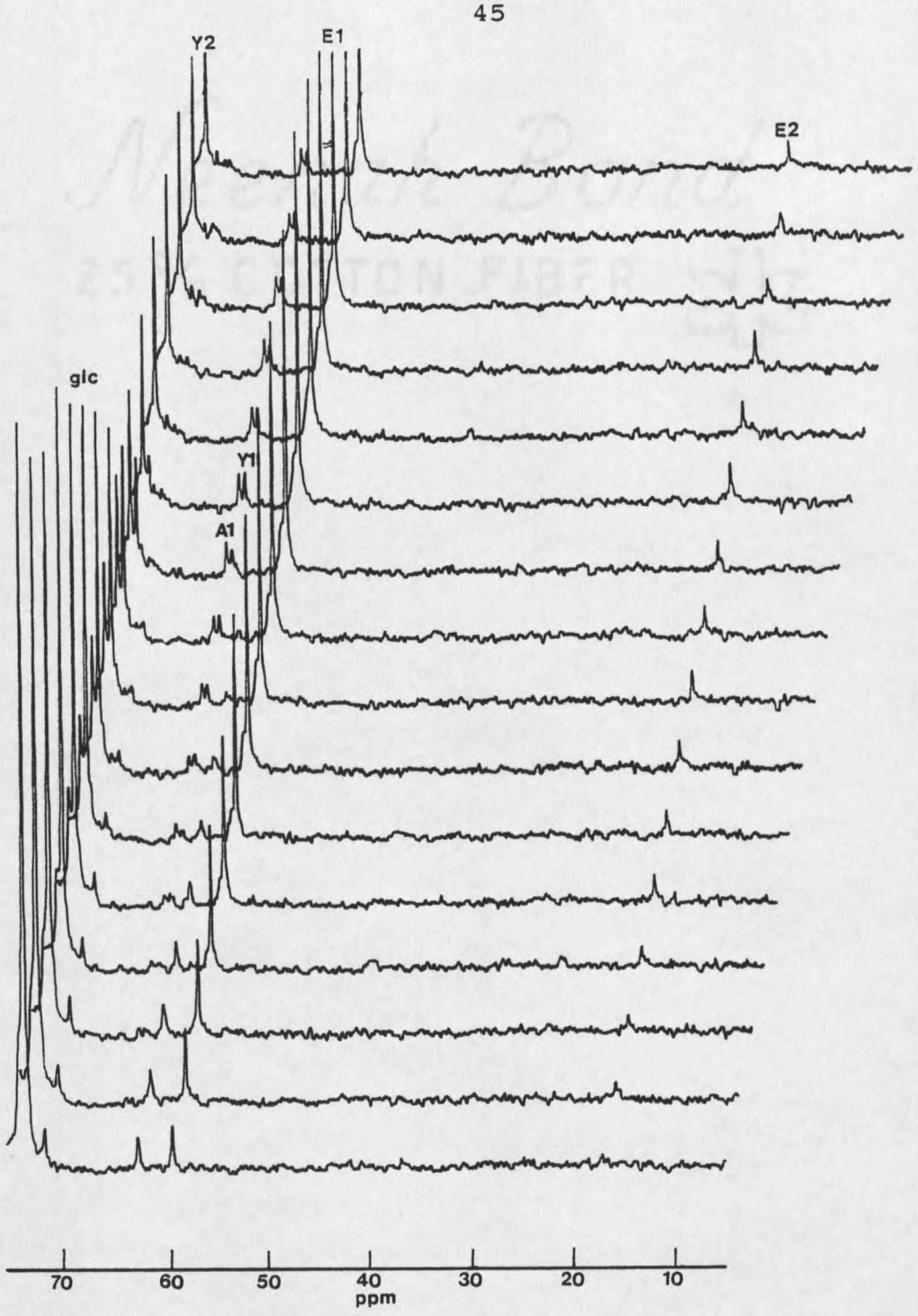

Figure 17. C-13 NMR spectra of the results of C. albicans strain 118 acting upon 2-C-13-glucose. Each spectrum represents a 15 minute accumulation. The abbreviations are the same as those used in Figure 13.

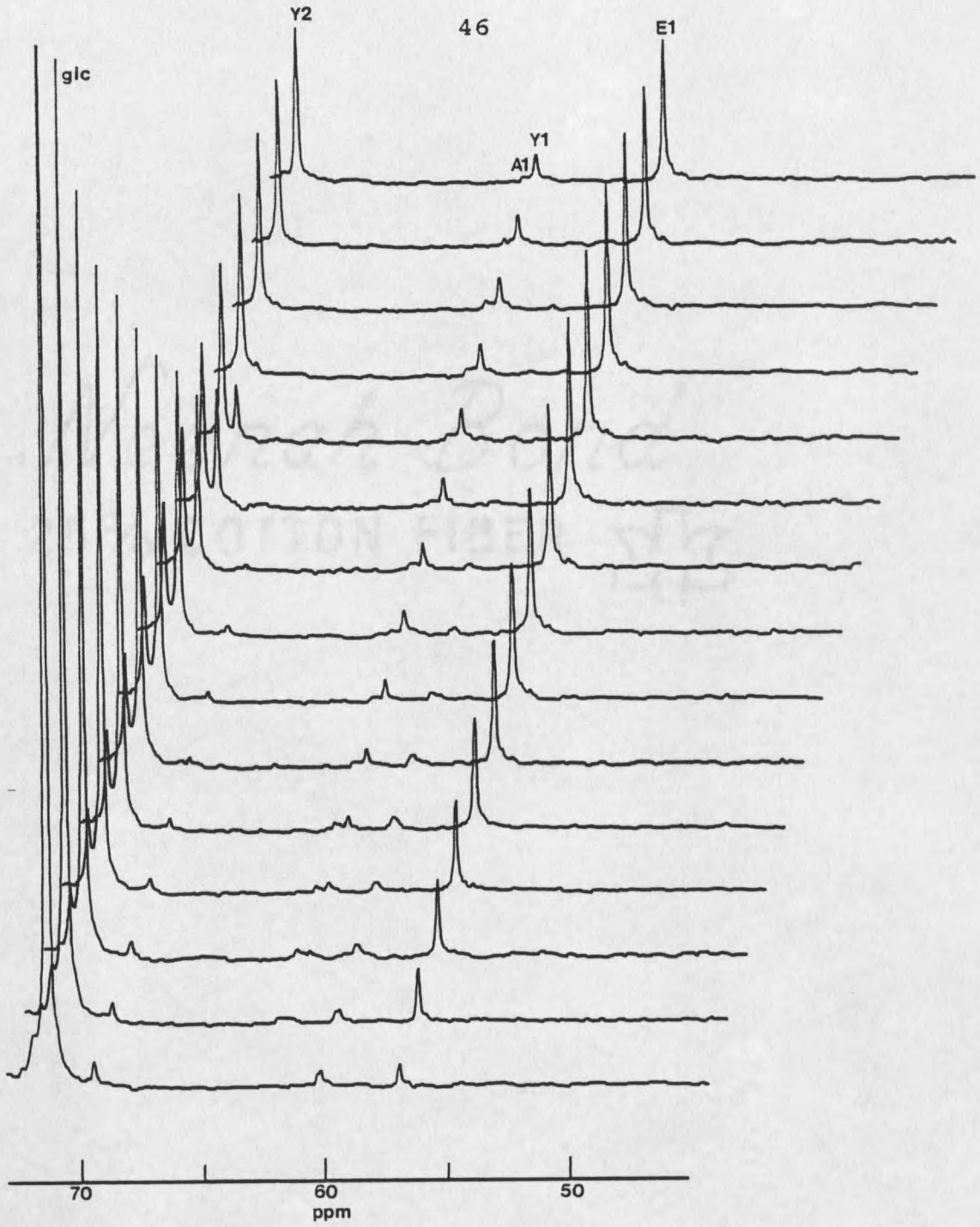

Figure 18. C-13 NMR spectra of the results of C. albicans strain 122 acting upon 2-C-13-glucose. Each spectrum represents a 15 minute accumulation. The abbreviations are the same as those in Figure 13.

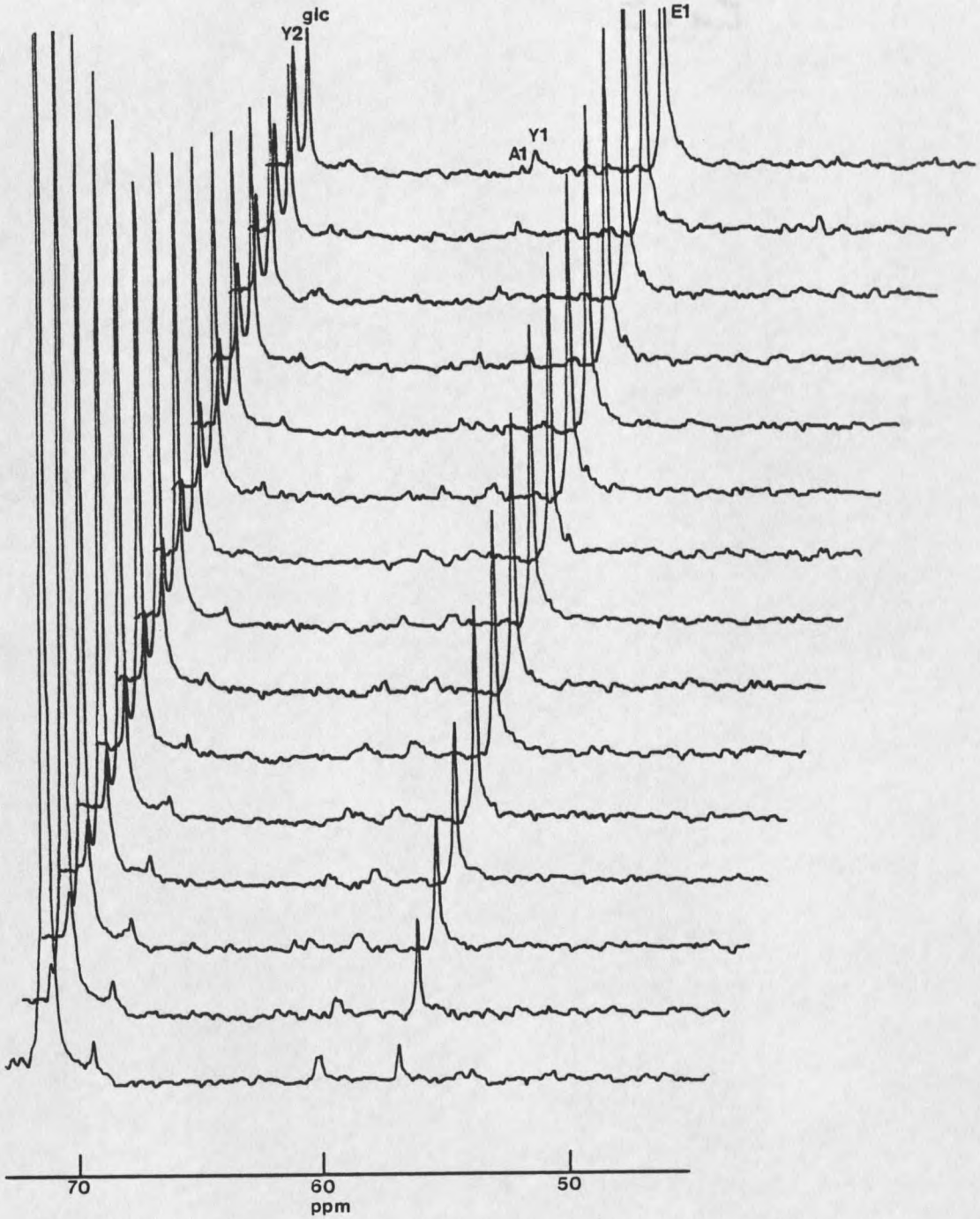

Figure 19. C-13 NMR spectra of the results of C. albicans strain 117 acting upon 2-C-13-glucose. Each spectrum represents a 15 minute accumulation. The abbreviations are the same as those in Figure 13.

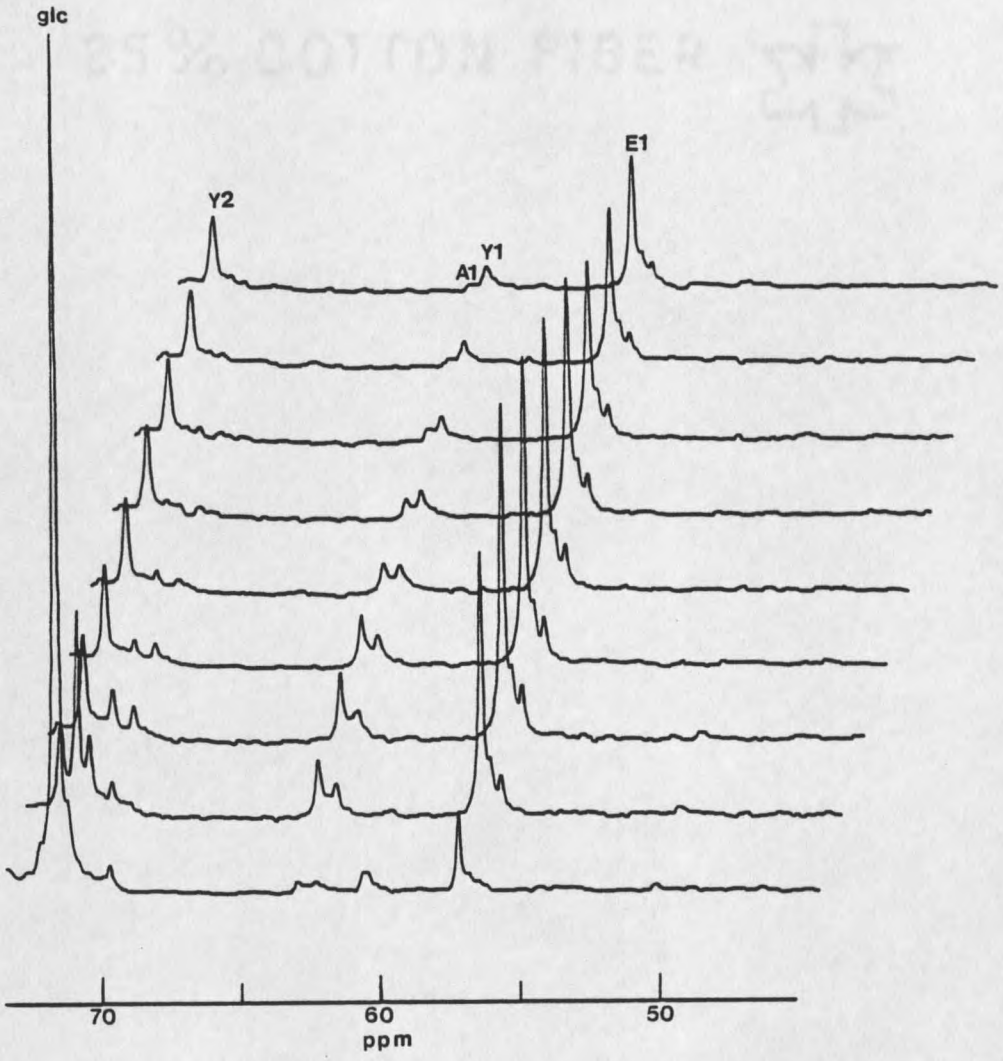

Figure 20. C-13 NMR spectra of the results of C. albicans strain 118 at a concentration of 10^9 cells/ml acting upon 2-C-13-glucose. Each spectrum represents a 15 minute accumulation. The abbreviations are the same as those in Figure 13.

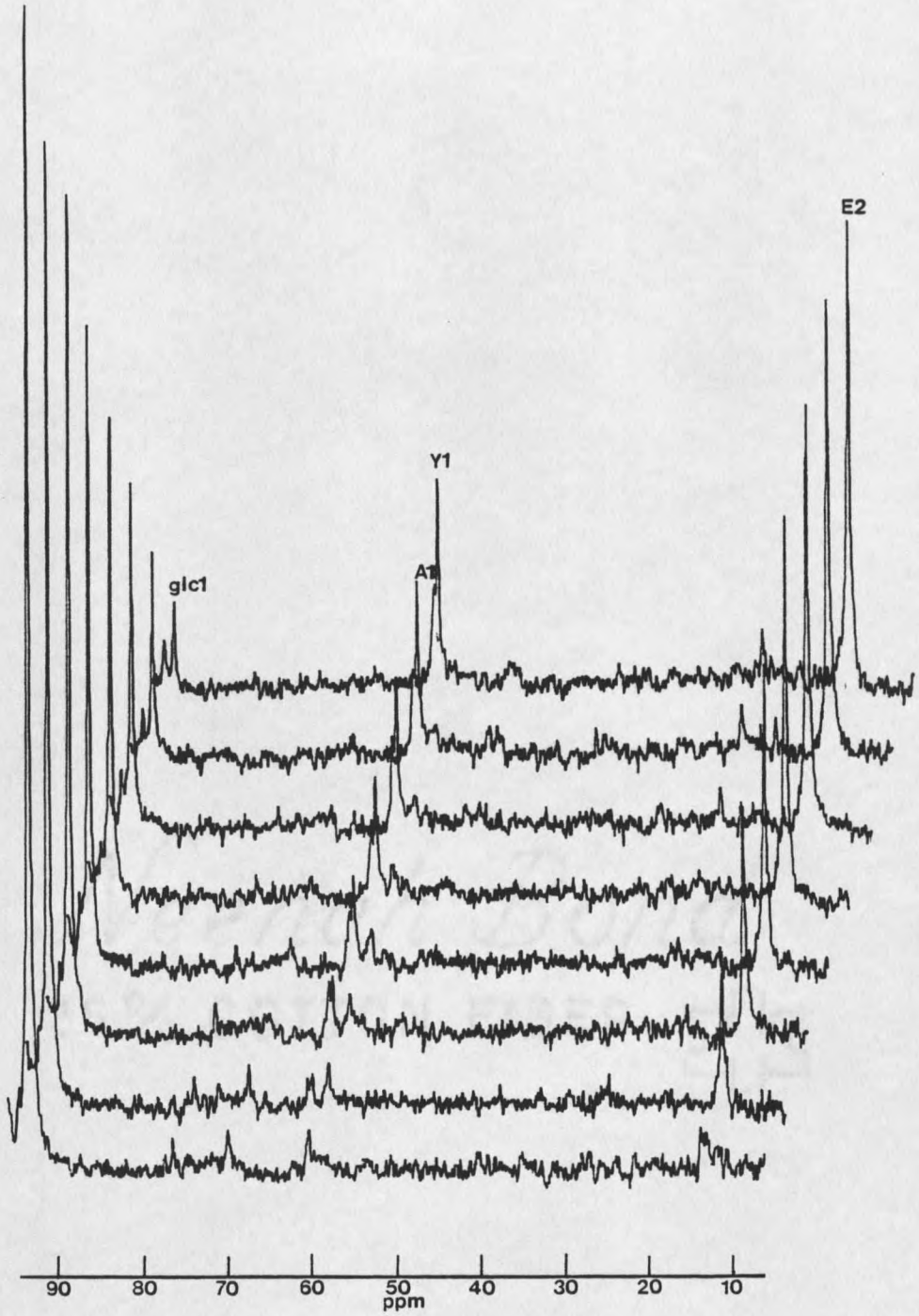

Figure 21. C-13 NMR spectra of the results of C. albicans strain 118 acting upon 1-C-13-glucose. Each spectrum represents a 15 minute accumulation. The abbreviations are the same as those in Figure 13.

higher than that of arabinitol. This experiment was duplicated only once as the cell metabolism changed shortly thereafter.

1-C-13-Xylose Experiments

The 1-C-13-xylose experiments reveal the presence of 1-C-13-glycerol and 1-C-13-arabinitol. Figure 22 shows the results of the experiment with strain 118, type I cells. The peaks from 65-77 ppm were the natural abundance peaks of the labeled xylose. The xylose experiments were successfully repeated three times.

Supernatant Liquid from 2-C-13-Glucose Experiments

Figure 23 shows the results of an 8-hour C-13 NMR accumulation of the cell-free supernatant liquid extracted from the 2-C-13-glucose experiment shown in Figure 15. The resonances at 74.6 and 72.6 ppm are due to the labeled carbons of the glucose as split by the anomeric carbon. The 2-C-13-β-glucose resonance at 74.6 ppm is truncated. The truncated resonance at 72.6 ppm is due to 2-C-13-glycerol. The 1-C-13-, and 1,3-C-13-glycerol peak is at 62.8 ppm. The 1-C-13-, 2-C-13- and 4-C-13-arabinitol resonances are at 63.4, 70.6 and 71.3 ppm, respectively. The resonances due to labeled ethanol are not present because the sample was freeze-dried during work-up. Figure 24 is the same experiment except the supernatant liquid was from type III cells.

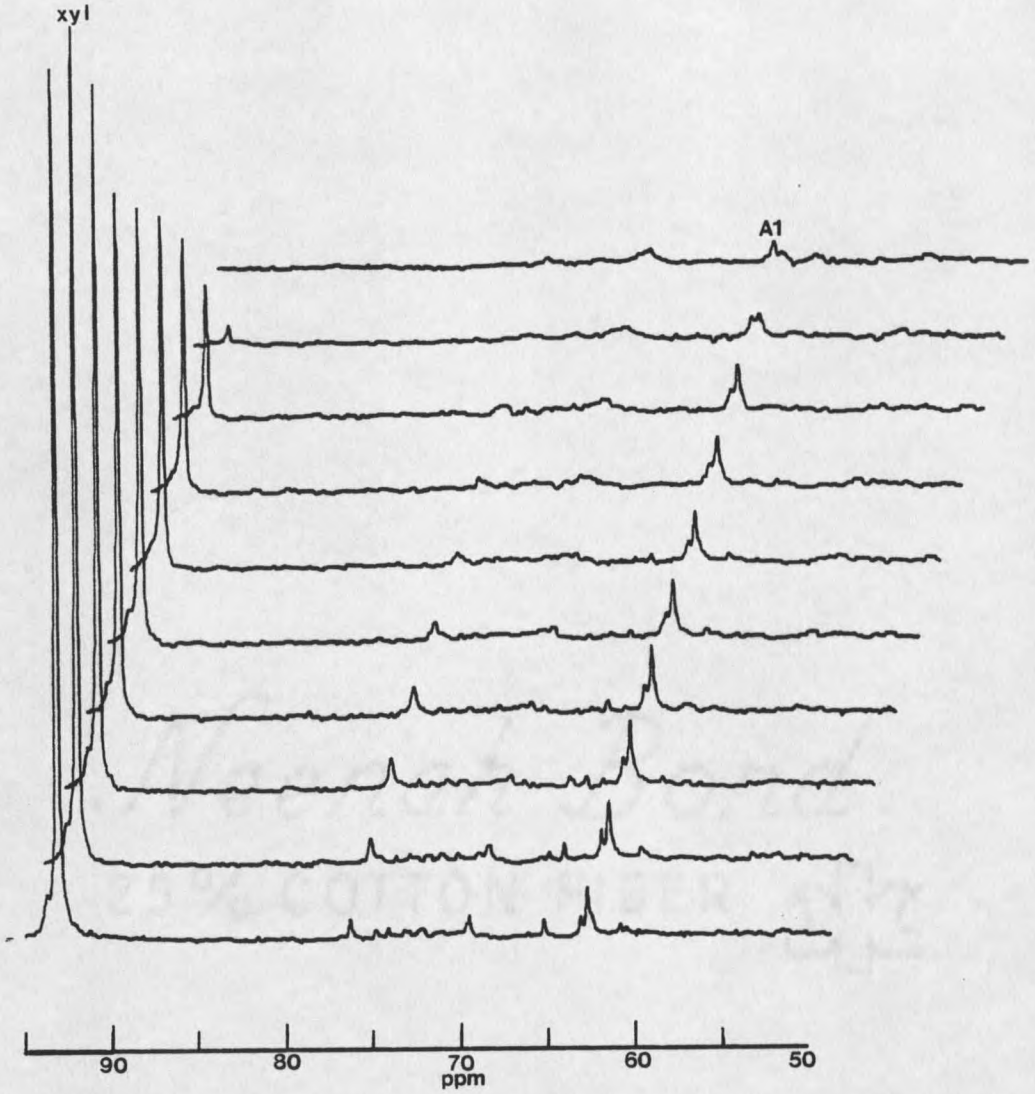

Figure 22. C-13 NMR spectra of the results of C. albicans strain 118 acting upon 1-C-13-xylose. Each spectrum represents a 15 minute accumulation. The abbreviations are the same as those in Figure 13.

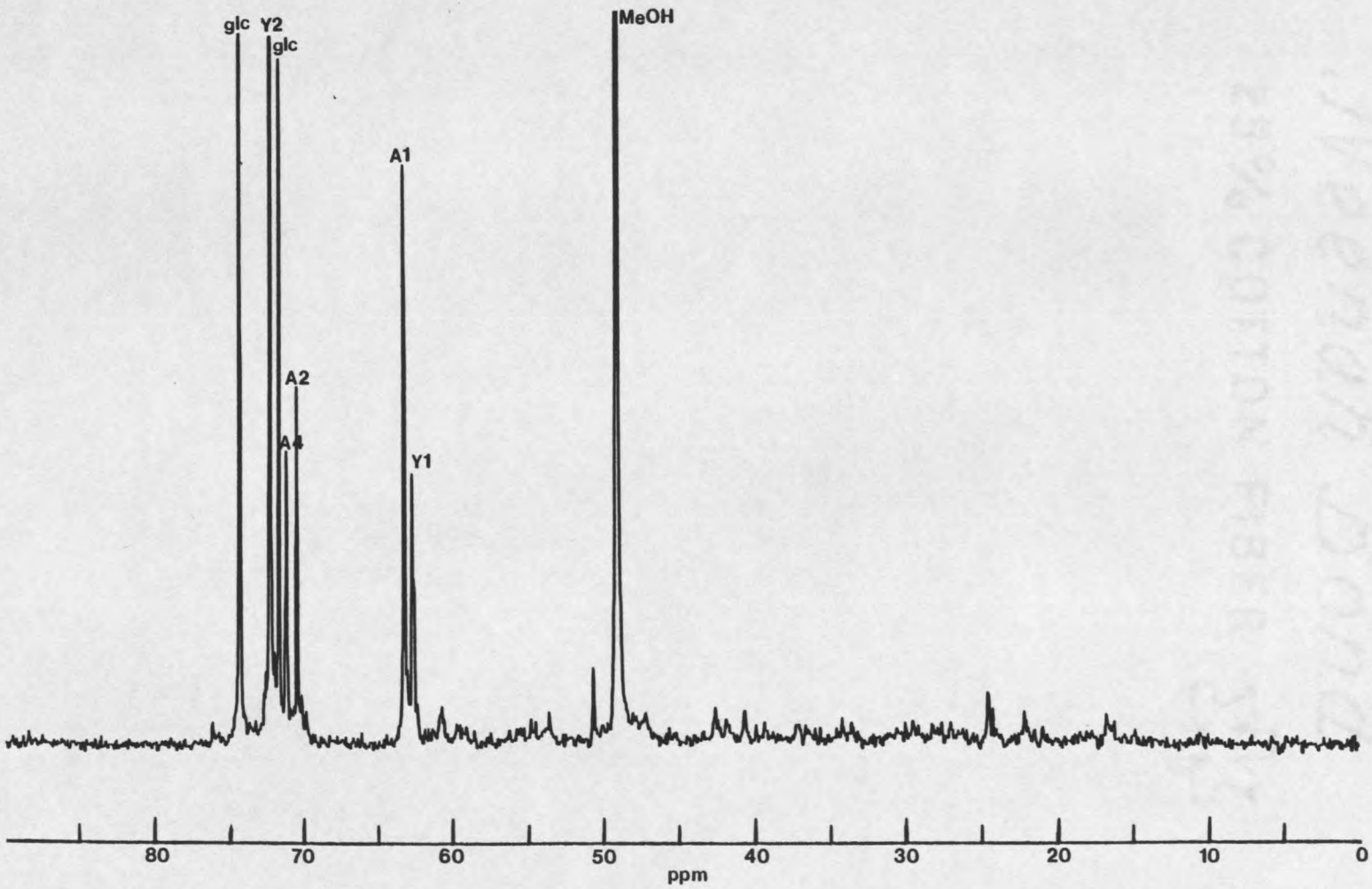

Figure 23. C-13 NMR spectrum of the supernatant liquid from type I yeast fed 2-C-13-glucose. Results from the experiment in Figure 15. The abbreviations are the same as those in Figure 13.

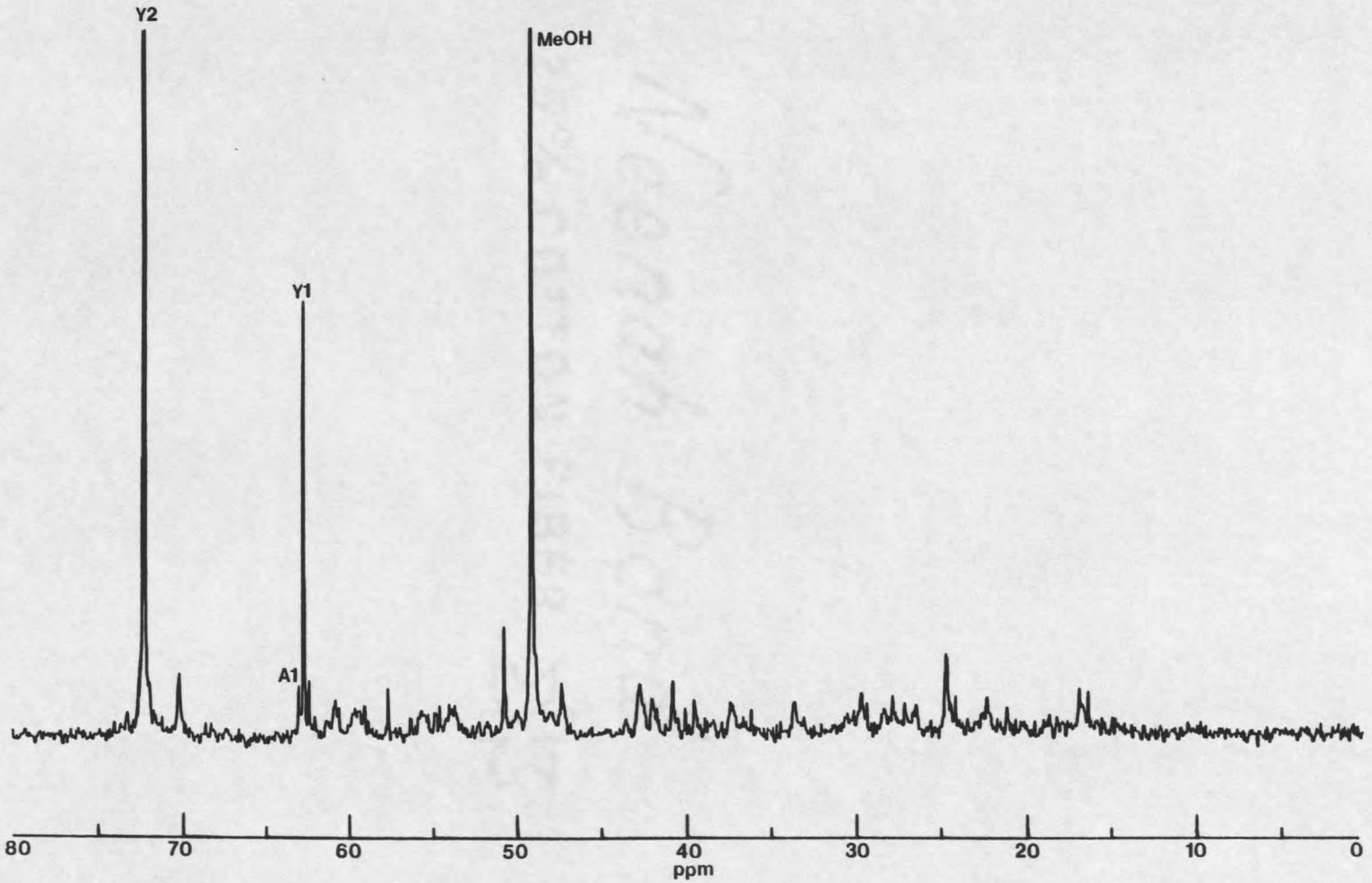

Figure 24. C-13 NMR spectrum of the supernatant liquid from type III yeast fed 2-C-13-glucose. The abbreviations are the same as those in Figure 13.

Table 2 shows the integrals of the resonances found in Figure 23 and from the supernatant liquid of the experiment shown in Figure 16. The integrals of both sets of data were scaled relative to the 2-C-13-glycerol peak to permit comparison. The percent contribution of 1-C-13-arabinitol to the total pool of labeled arabinitol was 48% and 47% for the samples shown in Figures 15 and 16, respectively, which can be rounded to 50%. The contributions from 2-C-13-arabinitol were 27% and 28% from the samples shown in Figures 15 and 16, respectively, which round to 25%. The contributions from 4-C-13-arabinitol were both 25%. The values for the arabinitol percentages are the 10% error margin that is reported for the Bruker peak picking routine. The ethanol resonances are absent from the supernatant liquid listing because the sample was freeze-dried before the spectrum was gathered.

Table 2. Integrals in arbitrary units of the resonances from the supernatant liquid from the experiments shown in Figures 15 and 16. Units have been scaled so the 2-C-13-glycerol peaks are equal to permit comparison.

	Integrals of Resonances		
Labeled Products	Figure 15	16	average
1-C-13-Arabinitol	0.33	0.46	0.39
2-C-13-Arabinitol	0.19	0.28	0.23
4-C-13-Arabinitol	0.17	0.25	0.21
1-C-13-Glycerol	0.14	0.16	0.15
2-C-13-Glycerol	1.0	1.0	1.0

Supernatant Liquid from 1-C-13-Glucose Experiments

The supernatant liquid from the 1-C-13-glucose experiment was analyzed to take advantage of the increased resolution available when the sample is not being bubbled with oxygen and the cells are absent. Figure 25 shows the results of an 4-hour run. The peaks at 63.4 and 63.3 ppm represent 1-C-13- and 5-C-13-arabinitol, respectively. The peak at 62.8 ppm is 1-C-13-glycerol. The 2-C-13-glycerol resonance is barely visible at 72.4 ppm. The 2-C-13-ethanol peak is at 17.1 ppm. Based upon the peak intensities, the ratio of the 1-C-13-arabinitol peak to the 5-C-13-arabinitol peak is 1.2:1.0. Intensities were used instead of integrals because of the strong overlap at the base of the peaks. The ratio is nearly the same as that for the two- and four-labeled arabinitol peaks from the two labeled glucose experiments. The 2-C-13-arabinitol peak to the 4-C-13-arabinitol integral ratio is 1.1:1.0 (Table 2).

Cumulative Spectra from 1-C-13-Xylose Experiments

While the supernatant liquid of the 1-C-13-xylose experiment was not analyzed, the results of addition of the spectra are shown (Fig. 26). Clearly visible are the expected one-labeled arabinitol and one-labeled glycerol peaks, and the peaks for two- and four-labeled arabinitol and for two-labeled glycerol. Other resonances are present, such as the C-2 peak of alanine at 16.8 ppm, but they do not affect the analysis of the polyol peaks.

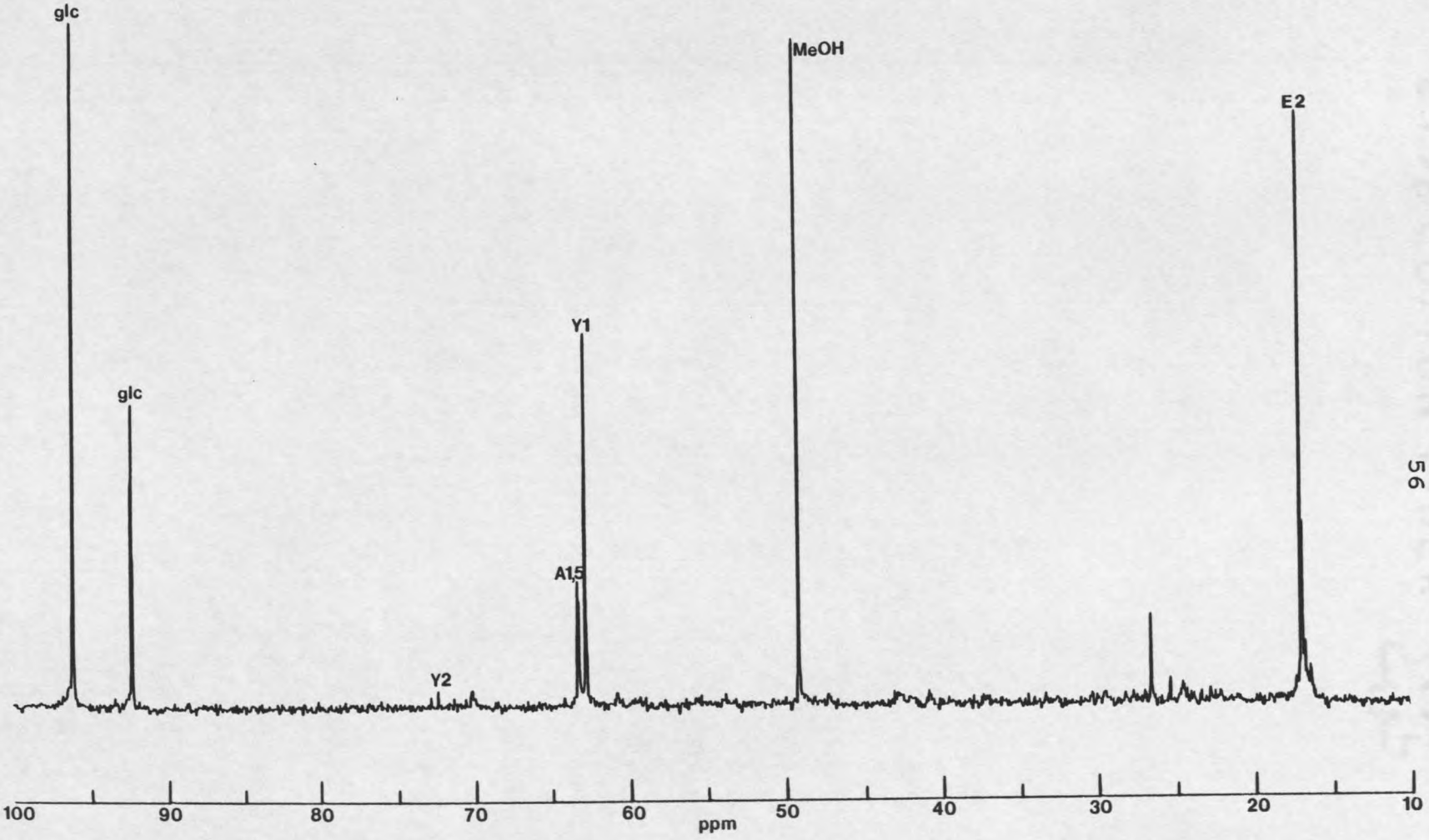

Figure 25. C-13 NMR spectrum of the supernatant liquid from type I yeast fed 1-C-13-glucose. Results from the experiment in Figure 18. The abbreviations are the same as those in Figure 13.

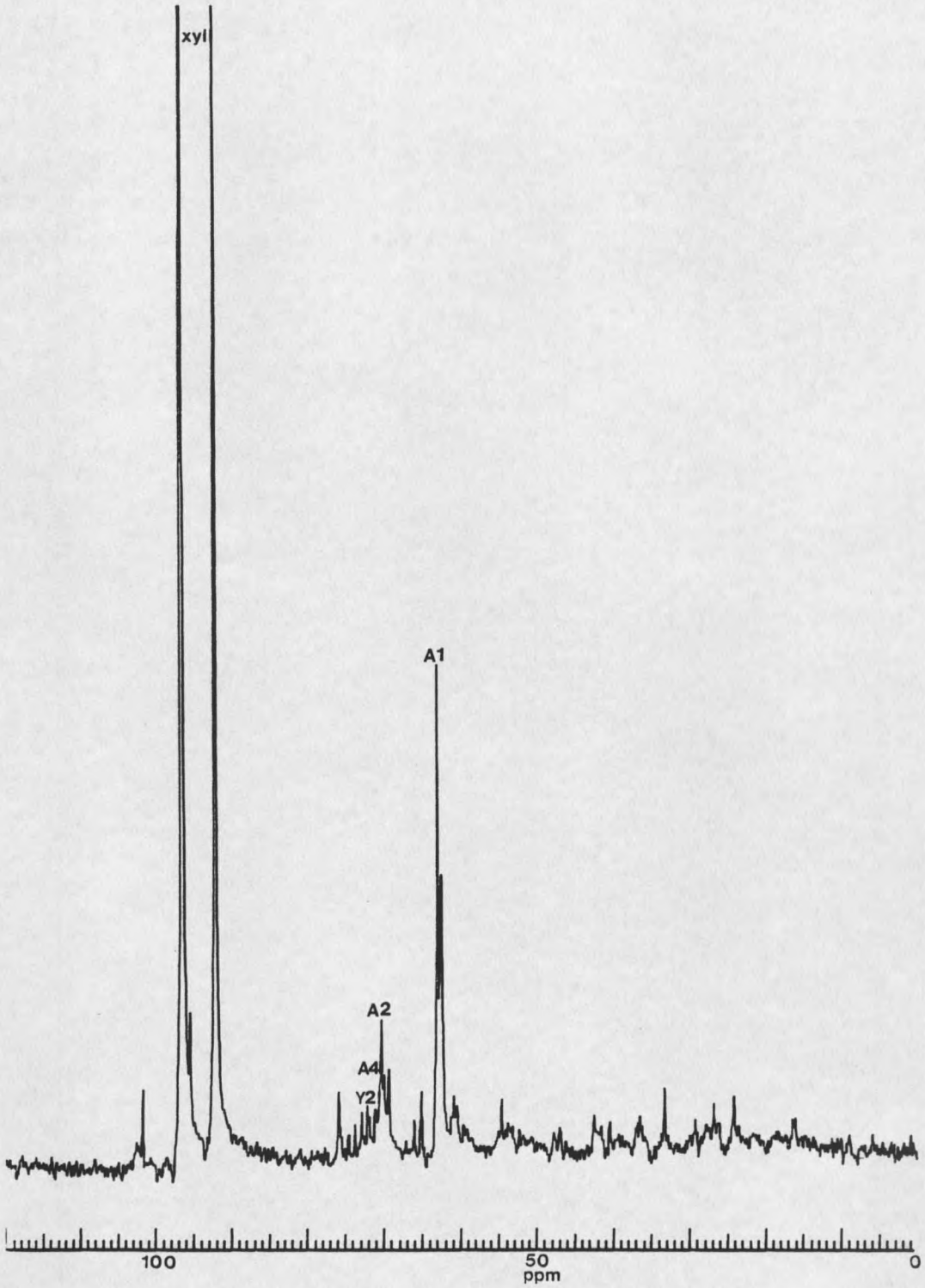

Figure 26. C-13 NMR spectrum of the accumulated files from the labeled xylose experiment shown in Figure 19. The abbreviations are the same as those in Figure 13.

Cell Extracts from 2-C-13- and 1-C-13-Glucose Experiments

The extracts of the cells show a slightly different pattern than the pattern found in the supernatant liquids. In the 2-C-13-glucose case, all of the arabinitol and glycerol resonances found in the supernatant liquid are clearly visible, but the relative integrals are not the same as those found in the supernate (Fig. 27). The 1-C-13-glucose experiment shows a similar result (Fig. 28). The 1-C-13 arabinitol is clearly visible, as is the 1-C-13-glycerol peak. However, the 5-C-13-arabinitol peak is a mere shoulder on the side of the peak due to the one labeled arabinitol. The resonances at 96.4 and 92.6 ppm are due to glucose while the resonance at 93.6 is unassigned.

Natural Abundance Classification

Several different yeast were examined by natural abundance C-13 NMR. C. tropicalis (Fig. 29) has very few defined resonances in the region from 0-58 ppm. Alanine and glutamate are visible at 16.4 and 50.9 ppm, and 27.2, 33.5 and 54.9 ppm, respectively. Glutamine may also be present as there is a peak at 26.5 ppm, but the 31.1 and 54.2 ppm resonances are either lost in noise or strongly overlapped by neighboring peaks. Glycerol is a low concentration polyol that may be present in C. tropicalis, with a resonance at 62.5 ppm that is barely separated from

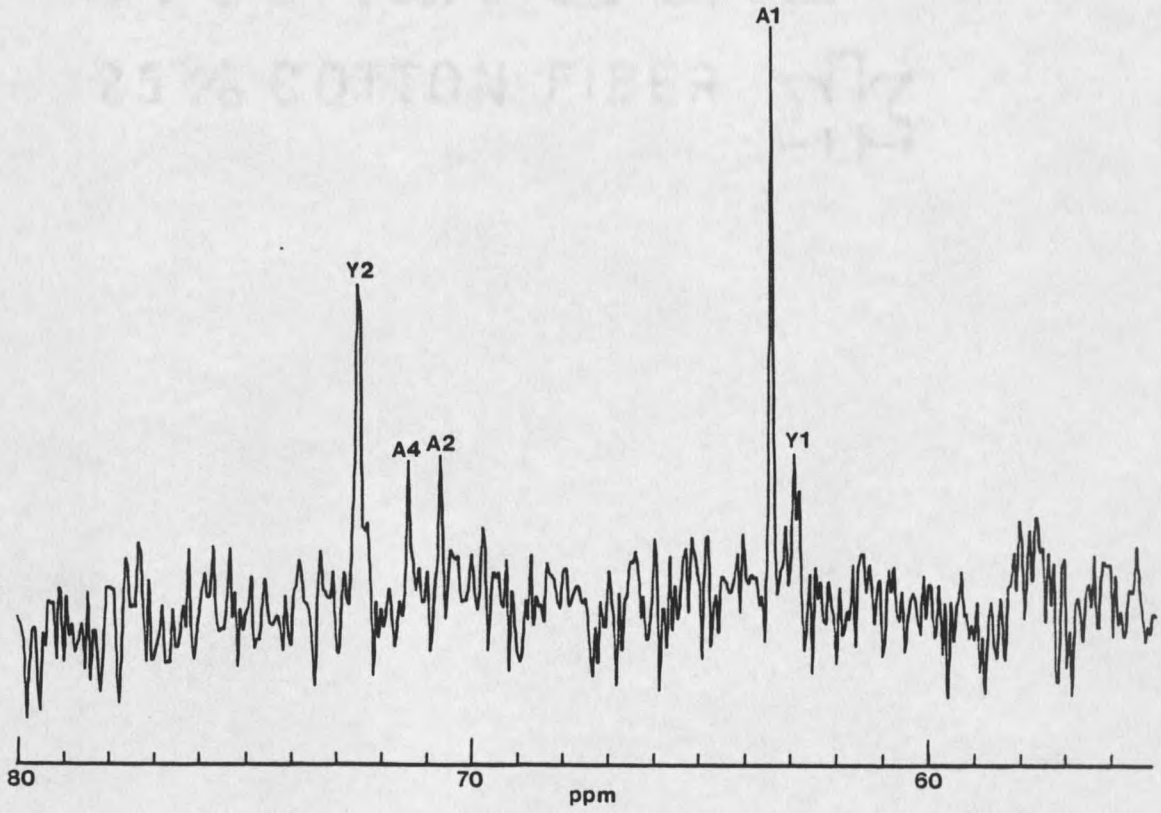

Figure 27. C-13 NMR spectrum of the cell extract from an experiment using strain 118 cells that were fed 2-C-13-glucose. The abbreviations are those used in Figure 13.

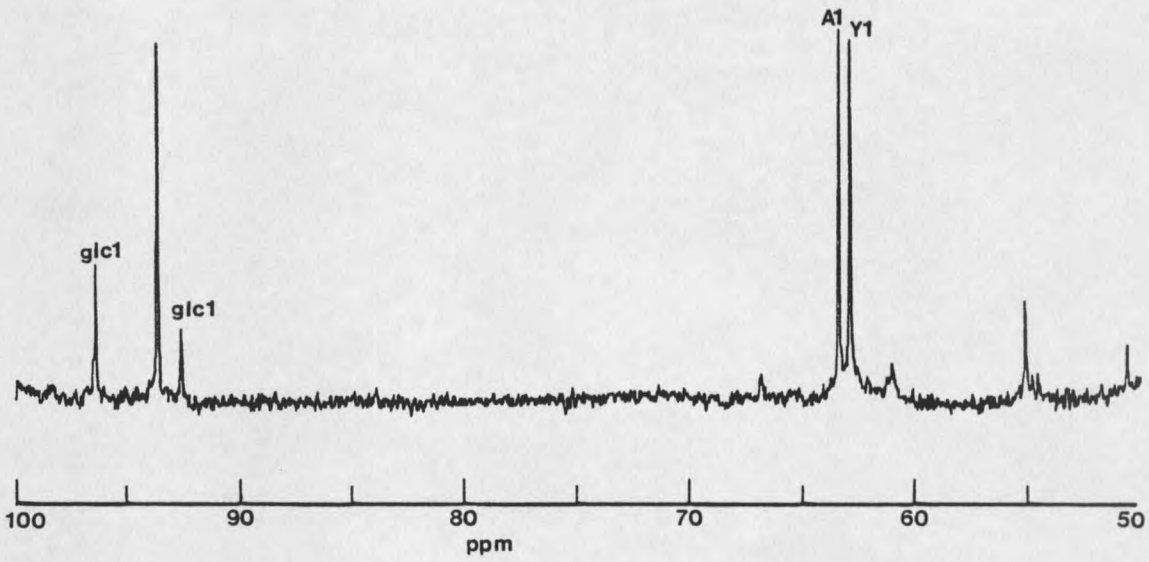

Figure 28. C-13 NMR spectrum of the cell extract from an experiment using strain 118 cells that were fed 1-C-13-glucose. Results from the experiment in Figure 18. The abbreviations are those used in Figure 13.

neighboring peaks, and a resonance at 72.4 ppm which overlaps with trehalose's peak at the same chemical shift. The major carbohydrate component appears to be trehalose.

C. pseudotropicalis (Fig. 29) has only glutamate and glutamine as identifiable resonances at 27.2, 33.5, and 54.9 ppm, and 26.5, 31.1, and 54.4 ppm, respectively. Glycerol is present at very small concentrations. Its peaks at 72.4 and 62.5 ppm barely rise above the baseline.

Torulopsis glabrata (Fig. 29) has resonances due to alanine at 16.3 and 50.6 ppm, ethanol at 17.1 and 57.5 ppm, glutamate at 27.2, 33.5 and 54.6 ppm, and glutamine at 26.7, 31.1 and 54.2 ppm. Proline is present at a low concentration. Its resonances at 23.9 and 46.1 ppm are easily distinguishable from the noise and other peaks, but the peak at 29.2 ppm is probably lost in the noise and other peaks around it. The peak at 61.1 ppm is probably present, but shifted to 61.3 ppm because of overlap with neighboring peaks. The resonances for trehalose are present at 93.3, 72.8, 72.4, 71.3, 69.9 and 60.7 ppm. These resonances indicate that trehalose is one of the major small molecules in T. glabrata.

C. guillermondii (Fig. 30) has resonances that are indicative of glutamate at 27.2, 33.5 and 54.9 ppm. There is a resonance at 50.6 ppm, which may indicate the presence of alanine, but the associated 16.4 ppm peak may be buried in the high level of background in the 10-20 ppm region.

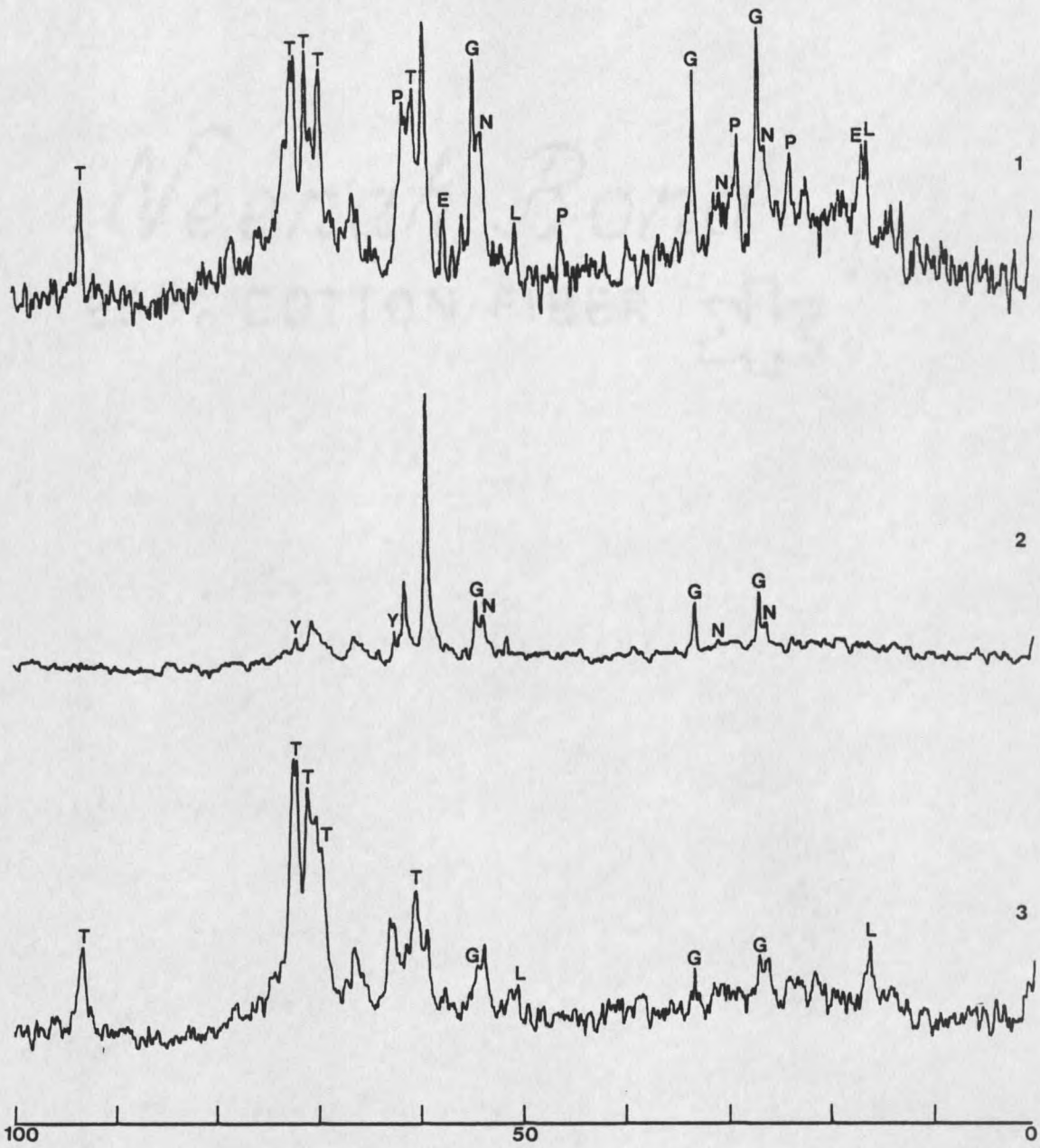

Figure 29. C-13 NMR spectra of 1) C. pseudotropicalis, 2) C. tropicalis and 3) Torulopsis glabrata. The abbreviations are the same as used in Figure 2.

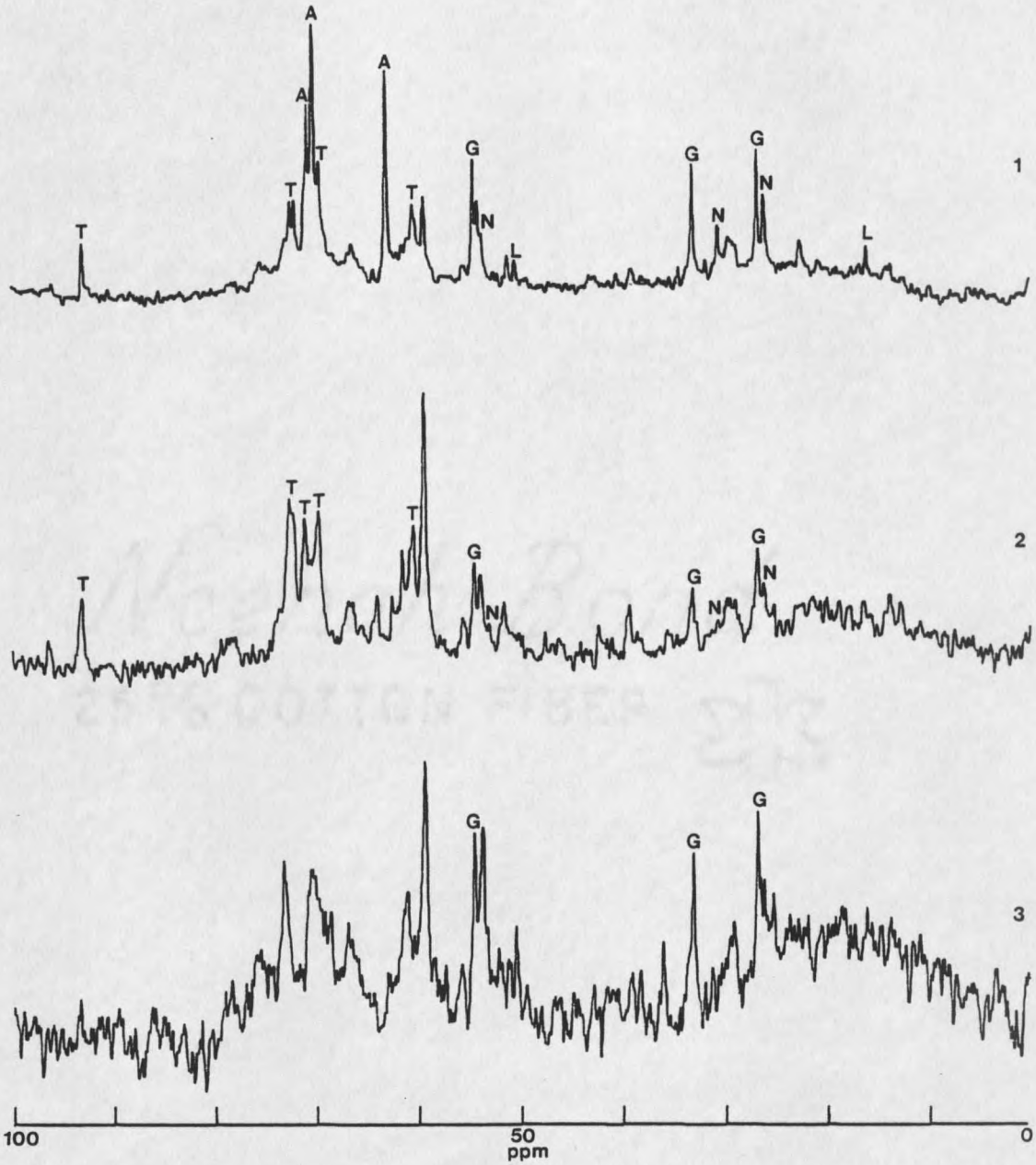

Figure 30. C-13 NMR spectra of 1) C. albicans, 2) C. krusei, and 3) C. guillermondii. The abbreviations are the same as used in Figure 2.

The major peaks at 61.2, 67.0, 68.7, 70.6 and 73.4 ppm have not been identified.

C. krusei (Fig. 30) has the resonances for glutamate and glutamine at 27.2, 33.5, and 54.9 ppm, and 26.7, 31.1, and 54.4 ppm, respectively. The spectrum is dominated by the resonance at 59.4 ppm. The carbohydrate region is characterized by the peaks due to trehalose. Glycerol is also present at a low concentration. The C. albicans spectrum is included for comparison to the other spectra (Fig. 30).

In the early stage of these investigations, we found that the natural abundance spectra obtained for a given strain were unique. Figure 31 demonstrates this result with the type I organism.

In an attempt to further define the classification of the strains of C. albicans, the relative intensities of several resonances were compared among the strains. The height of the C-2, C-4 peak of arabinitol was set at 4.0, and the height of the C-1, C-1' peak of trehalose and the C-3 peak of glutamate were measured relative to it (Table 3). The mean(x) and pooled variance estimator(s^2) were calculated. These values were used to test the hypothesis that the means of the two populations of cells were the same, H_o: $\mu_1=\mu_2$. A one-tailed t-test with a confidence limit of 99% was used.

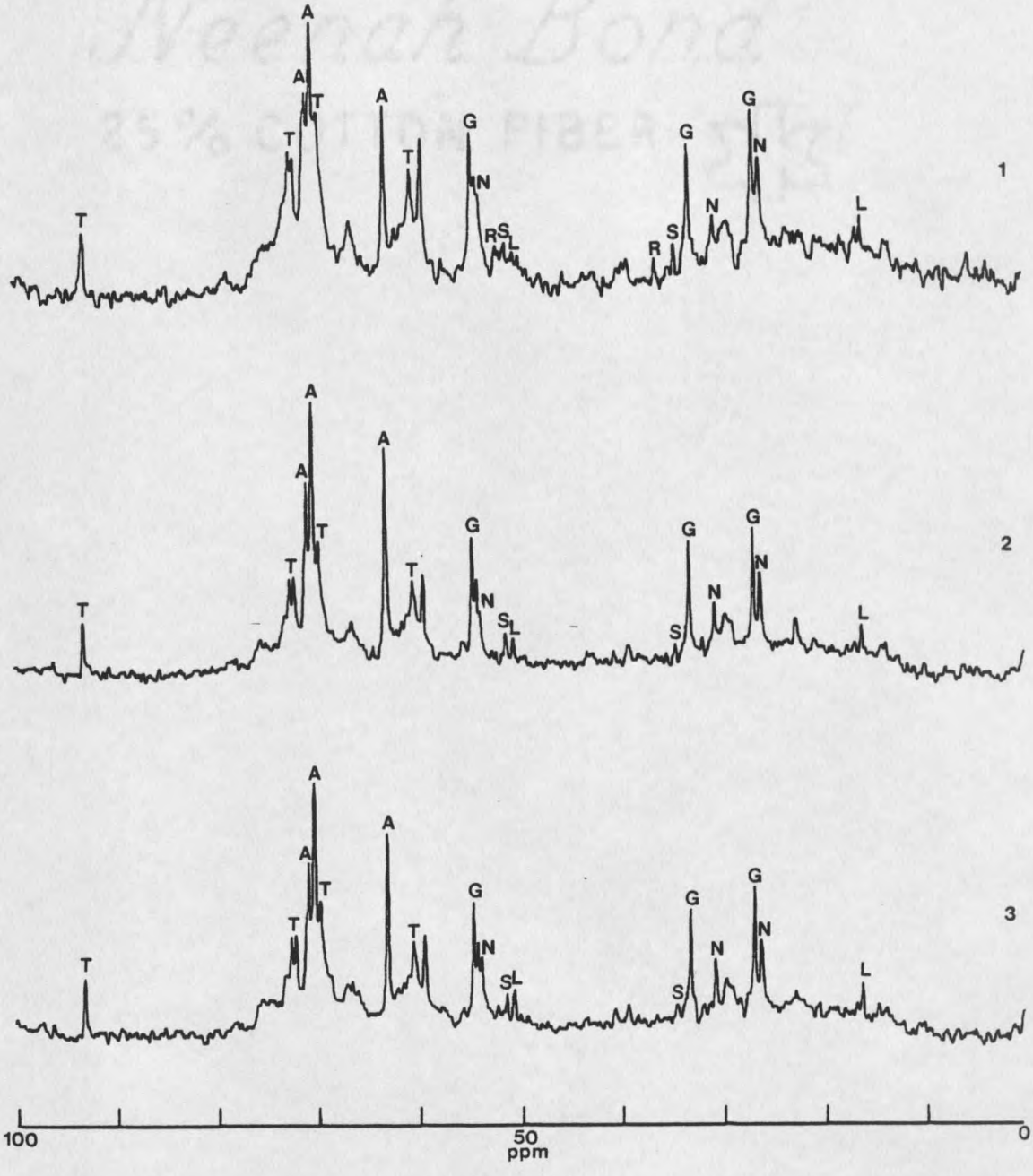

Figure 31. C-13 NMR spectra of C. albicans type I strain 118 showing similarities and differences of the same strain at various times, 1) Oct. 16, 2) and 3) Aug. 4. The abbreviations are the same those used in Figure 2.

Table 3. A listing of the intensities of the C-1,C-1' trehalose and C-3 glutamate resonances relative to the overlapping C-2, C-4 resonances of arabinitol.

Type I Strains

Type	Strain #	Date	Trehalose C-1,C-1'	Glutamate C-3
I	16	8/4	1.17	2.47
	104	8/7	1.34	2.81
		8/10	1.22	2.60
	105	8/4	1.34	2.59
		8/4	1.30	2.31
	112	8/10	1.08	2.14
	113	8/13	0.91	2.12
	118	8/4	0.89	1.83
		8/4	0.76	1.81
		8/7	1.15	2.08
		8/10	1.02	2.72
		8/13	1.06	2.59
		8/14	1.34	3.07
		9/3	1.48	2.48
		9/23	1.50	3.17
	129	10/1	1.08	1.80
	130	10/1	1.06	1.68
	131	10/1	1.24	2.59
	138	8/13	0.71	2.51
	149	8/13	1.00	2.97
	158	10/7	1.47	2.86
	159	10/7	1.24	1.81
	160	10/7	1.45	2.84
	161	10/7	1.60	3.15
	163	10/7	1.30	1.56
	164	10/7	1.53	1.99
		10/7	1.06	1.17
II	109	8/4	0.15	1.26
		8/14	0.08	1.29
	113	8/10	0.55	1.48
		8/10	0.42	1.43
	122	8/10	0.15	0.66
		8/13	0.24	0.73
		9/21	0.35	0.94
		10/18	0.28	0.84
		10/21	0.17	0.74
	135	8/4	0.57	1.52
		8/7	0.64	1.40
	147	8/7	0.59	1.28
		8/7	0.50	1.48
		8/13	0.40	1.73

$$t = \frac{x_1 - x_2}{s_d}$$

$$s_d = \frac{E\,(x_{1j} - x_1)^2 + E(x_{2j} - x_2)^2}{n_1 + n_2 - 2}$$

Trehalose calculations:

$x_1\ \mu = 1.19$ (type I cells)

$E(x_{1j} - x_1)^2 = 1.43$

range = 0.76-1.53

$x_2\ \mu = 0.36$ (type II cells)

$E(x_{2j} - x_2)^2 = 0.46$

range = 0.08-0.64

$$s_d^2 = \frac{1.43 + 0.46}{27 + 14 - 2} = 0.0485$$

$s_d = 0.22$

$$t = \frac{1.19 - 0.36}{0.22} = 3.77$$

$t_{.005,40} = 2.704$, therefore the hypothesis, H_o, is rejected.

Glutamate calculations:

$x_1\ \mu = 2.39$ (type I cells)

$E(x_{1j} - x_1)^2 = 7.27$

range = 1.17-3.45

$x_2\ \mu = 1.20$ (type II cells)

$E(x_{2j} - x_2)^2 = 1.57$

range = 0.66-1.57

$$s_d^2 = \frac{7.27 + 1.57}{27 + 14 - 2} = 0.227$$

$$s_d = 0.48$$

$$t = \frac{2.39 - 1.20}{0.48} = 2.48$$

$t_{.01,40} = 2.423$, therefore the hypothesis, H_o, is rejected.

As shown by the range of values measured for the glutamate resonances, there was some overlap between the type I and type II cells, but there was no overlap in the trehalose case. Although the t-test indicates that a judgement of type can be based upon either test, the trehalose test has a higher degree of confidence.

DISCUSSION

Natural Abundance Spectra of Candida albicans

Natural abundance C-13 spectra obtained have two experiment delineating characteristics. They are the natural linewidths of the peaks and the S/N of the samples. The linewidths of the spectra shown are on the order of 10-15 Hz. This results from the use of a S/N enhancement technique that sacrifices resolution for increased signal intensity. The natural linewidths were from 5-10 Hz. This is more than ten times broader than the widths observed in C-13 NMR spectra under ideal conditions. Several factors contribute to the broadening. They are the homogeneity of the sample's magnetic field, viscosity of the sample, paramagnetic materials in the sample, and the homogeneity of the sample.

The homogeneity of the magnetic field in which the sample sits has a profound effect upon linewidths. Three different methods of optimizing homogeneity were used in these experiments. The first method used a coaxial tube of D_2O with a tetramethylsilane (TMS) capillary inside to act as a reference. The second used an external $CDCl_3$ source as both a lock and a reference. The last consisted of

adding D_2O directly to the cells in the tube. All three methods gave comparable S/N and linewidths.

The viscosity of the medium that a compound is in has an effect on the linewidth of the obtained resonances. The denser the medium, the broader the lines. This results from an increase in the amount of relaxation the C-13 nuclei obtain from collisions with adjacent molecules. The faster the relaxation, the wider the observed resonances. The cytosol of the cells is probably a fairly viscous medium (57).

Cells can contain enough paramagnetic material to increase the relaxation rate of the C-13 nuclei. The majority of this material takes the form of the various paramagnetic metal-containing proteins. Norton, MacKay and Borowitzka found that the paramagnetic species of Mn^{+2} and Cu^{+2} could have a measurable impact on the linewidths of the solutes in the cells (57). Fabry and San George published a warning concerning the magnetic susceptibility changes associated with studies of red blood cells (58). However, both of these reports dealt with organisms that have high concentrations of paramagnetic species. The first group worked with a cyanobacterium and an alga, both of which have high concentrations of Mn^{+2} due to the requirements of the photosynthetic enzymes. The second group worked with red blood cells, which have a high concentration of Fe^{+2} in the hemoglobin. Although yeast

cells do not have the concentrations of paramagnetic ions found in the previous samples, there will still be some contribution from such ions.

One of the largest contributors to the linewidths is the fact that all the cells are not homogeneous. The existence of these micro-environments will vary the exact frequency at which a peak will resonate. The source of the micro-environments can be little more than simple changes in the pH, a range of viscosities, or changes in magnetic susceptibility in or between the cells. Since the organisms are in a cell pack during the experiment, the magnetic susceptibility difference between the cells and surrounding medium will have only minimal effects.

The signal-to-noise ratio that can be obtained from an NMR spectrum is the other defining characteristic of the natural abundance experiment. There is a trade off involved in increasing the signal-to-noise ratio of a spectrum. Given the detection limits of the Bruker WM-250 and a half hour to do an experiment, it is barely possible to detect a compound at a 5 mmolar concentration. This was confirmed by measuring the intensities of known concentrations of arabinitol and alanine (results not shown). To detect a concentration one-half of that would require approximately four times the amount of time. This occurs because while the signal intensity increases with the number of acquisitions (N), the noise increases with

the square root of that number. The signal to noise ratio then becomes $N/N^{1/2}$, or just $N^{1/2}$. Recent advances in spectrometer design have increased detection limits by a factor of four or more, and would therefore allow these experiments to be done in less time or with greater detail for the same amount of time.

An additional characteristic concerns the sample. A cell slurry of E. coli requires eight hours to obtain S/N comparable to that of the yeast samples done in half an hour. The mycelial form of C. albicans also gave poorer S/N than the yeast forms of the cells (65). The differences may be due to the total amount of sample volume displaced by the cell walls and other materials that cannot be detected by solution NMR, this volume being larger in the bacterial and the mycelial cells.

Association of the spectral resonances present with the appropriate compounds is the biggest challenge in natural abundance C-13 spectroscopy. The method used to determine this was the comparison of the resonances found in the spectra to external standards. This is not unambiguous however. The overlap of resonances and the varying concentrations of the compounds present can hinder identification. This can be especially true if the compound has a low concentration in the sample and some resonances become obscured in clusters of higher concentration compounds. Given the lack of certainty

associated with this method, secondary evidence was used to support a given assignment. This included comparisons of the same compound to those reported in similar systems or to reports of the compound being detected in C. albicans.

Major Compounds Present in C. albicans

The major compounds of C. albicans visible in an NMR experiment are arabinitol, glycerol, trehalose, glutamate, glutamine, alanine, and proline. Arabinitol has been found in other yeasts and fungi (59,60,61,62) and also in C. albicans (53). Trehalose is a common carbohydrate found in many yeast. Shulman reports its existence in Saccharomyces cerevisiae (22).

The occurrence of the amino acids in the cells can be correlated to work that used standard extraction techniques (69). Using the results Sullivan **et al** obtained from cells of C. albicans, they detected the concentrations of many of the amino acids to be 1-30 mmolar. In stationary cultures, the concentration of glutamate and glutamine in those cells was approximately 5.7 and 4.4 mmolar, respectively. Sullivan **et al** found the concentrations to be 10.9 mmolar for glutamate and 4.7 mmolar for glutamine in exponential cells. Alanine's concentration was 1.1 mmolar in the stationary cells and 4.3 mmolar in the exponential cells. They did detect some compounds in concentrations lower than it is possible to see using the NMR. An example would be valine at 0.7 mmolar concentration in the exponentially-

grown cells. They also showed arginine's concentration to be 9.8 mmolar, which should have been easily detected in these experiments. However, in our studies some compounds had NMR detectable concentrations whereas in Sullivan's study the concentrations were too low to be detected. Proline and methionine were probably examples of this. These differences were probably due to the different media and strains of C. albicans used in their experiments as compared to mine. They used one ATCC cell line and two clinical isolates. Since all of the strains of C. albicans used in my experiments were clinical isolates, it is not likely that the strains were identical. It should also be noted that the concentrations estimated from the NMR peak heights appear to be in more agreement with the exponential phase results than the stationary phase results, even though both sets of experiments used stationary cultures that were 24 hours old.

Natural Abundance C-13 NMR Arabinitol Studies

The production of arabinitol by C. albicans has been used by several researchers as the basis for the clinical diagnosis of disseminated candidiasis (46-52). Since the three groupings of strains have widely different internal stores of arabinitol, I wanted to know if these differences were maintained under growth conditions that more closely resembled those in a tissue. In the first series of experiments, the cells were grown in a tissue culture

medium (M199, M. A. Bioproducts, Baltimore, MD.) (results not shown). Under these conditions, all of the types of cells had increased stores of arabinitol. Both M199 and SAAMF were chemically defined media. A comparison between the ingredients of the two showed that one of the major differences was the complex salt mixture added to the M199. Since growth on GYEP had been done earlier (65) and M199 without its salt mixture was not readily available, experiments were done using GYEP as the base medium and supplementing it with saline. Type I, II and III strains were grown on GYEP supplemented with 0.075, 0.150, 0.300 and 0.600 N NaCl.

The response of C. albicans to increasing osmolarity of the medium can be divided into two phases. The first was that elicited from the organisms at the lower concentrations of salt and consisted of an increased amount of arabinitol stored in the cells. A second feature of this phase was the absence of the peaks due to the presence of trehalose. This occurred at the lowest concentration of salt tested, 0.075 N. The disappearance of trehalose was probably due to the lessened amount of cellular energy being put into its production. Since trehalose is a disaccharide, it would be possible to make two molecules of arabinitol instead of one molecule of trehalose.

The second phase occurred at 0.600 N saline for the type I cells, and at 0.300 and 0.600 N for the type II and III

cells. At these salinities, noticeable concentrations of glycerol were noted in the cells. Its production was switched on at a salt concentration slightly less than 0.300N for the type II and III cells and just below 0.600N for the type I cells. Production is very active in type II and III cells growing at 0.600 N NaCl. The production of glycerol may be favored at the higher salinities because it is a smaller molecule; more of them can be produced per input of substrate. Given the salinity range studied, it was not known if the arabinitol production is shut down in favor of glycerol production or if the glycerol is produced from the present stores of arabinitol.

C. albicans response to increasing osmotic stress is not unique. The sugar-tolerant yeast Saccharomyces rouxii was found to accumulate arabinitol in response to an increase in the osmolarity of its environment (63). In this same study, S. cerevisiae, a non-sugar-tolerant yeast, did not respond to the high solute concentrations with the production of arabinitol. Debaryomyces hansenii is a halotolerant yeast that accumulates arabinitol and glycerol in response to an increasingly saline environment (64).

The growth temperature experiments were related to the saline experiments because C. albicans responded to this stress in a similar manner. They demonstrated that the arabinitol accumulation was under some form of temperature regulation. As the temperature decreased, the arabinitol

production increased. Since shifting the temperature of the medium is a common way of inducing germination (65), this observation suggests that all of the changes observed in the metabolism of C. albicans during germination induced by a temperature shift may not be due to the germination event itself.

Enriched Studies of Arabinitol Metabolism

The natural abundance studies of arabinitol production by C. albicans provided the background needed to study that production using labeled C-13 compounds. Glucose labeled in either the one position or the two position and xylose labeled in the one position with C-13 provided the compounds necessary for the investigation. The growth medium used was 0.5% yeast extract and 1.0% peptone supplemented with 0.3N NaCl. Although this amount of salt was greater than physiological saline, it was chosen because it maximized the amount of arabinitol produced by the cells in the natural abundance experiments. To this mixture 10mg of labeled sugar was added. The final concentration of glucose was 27.8 mM. The growth temperature was 28°C, again chosen because it maximized arabinitol production. The cells were 24 hrs old. Although other experiments on yeast metabolism used cells in the exponential growth phase (16,20), this proved unsatisfactory with C. albicans. Under the experimental

conditions, younger cells had a tendency to germinate in the NMR tube. Germinating cells are extremely sticky, tending to cling to the sides of the NMR tube in a large clump, resulting in an anaerobic culture. These anaerobic cultures did not produce detectable levels of arabinitol.

The labeled-substrate experiments revealed the probable pathways used by C. albicans to make its arabinitol, glycerol and ethanol.

Metabolism of 2-C-13-Glucose

When fed 2-C-13-glucose the cells produced primarily three different arabinitols labeled in the one, two and four position. Glycerol labeled in the one and two positions was produced. Theoretically, 1,3-C-13- and 3-C-13-glycerol could have been produced, but there was no way to differentiate its resonances from those of the 1-C-13-glycerol using the available resources. Since the metabolic source of all three glycerols is virtually the same, a separate discussion of each will not be done. The last labeled product of high concentration that was produced was ethanol labeled in the one and two positions.

Metabolism of 2-C-13-Glucose to 1-C-13-Arabinitol, 2-C-13-Glycerol, and 1-C-13-Ethanol

The one-labeled arabinitol, two-labeled glycerol and one-labeled ethanol were produced by the oxidative portion of the pentose phosphate pathway and the Embden-Meyerhoff-Parnass pathway (Fig. 32).

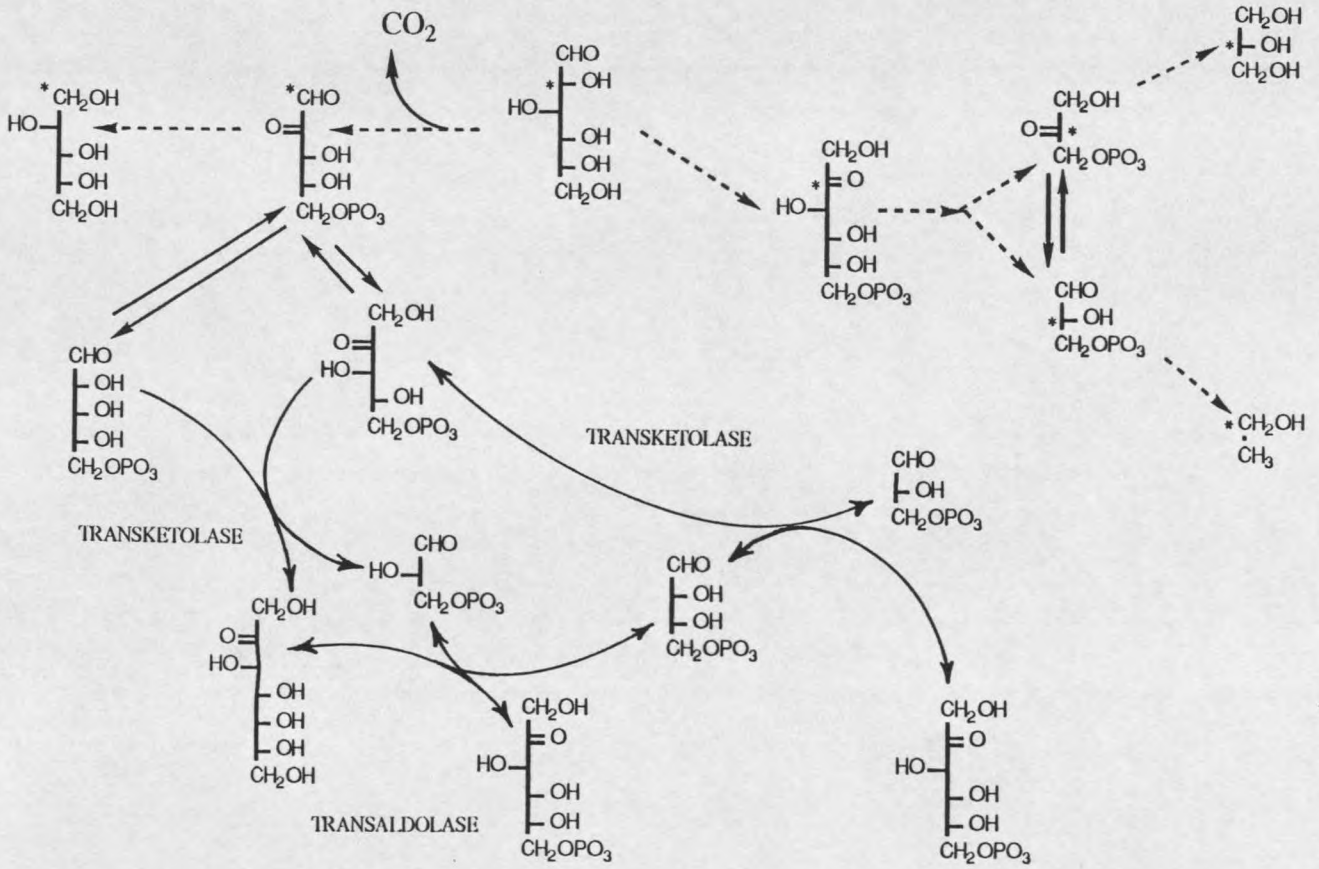

Figure 32. Pathways used by C. albicans to produce 1-C-13-arabinitol, 2-C-13-glycerol and 1-C-13-ethanol.

Metabolism of 2-C-13-Glucose to 1-C-13-Glycerol and 2-C-13-Ethanol

The one-labeled glycerol and the two-labeled ethanol were derived from the products from one pass through the pentose phosphate pathway. The first pass through the pentose phosphate pathway by 2-C-13-glucose could give fructose-6-phosphate labeled in the one position. This fructose would then generate one-labeled glycerol and two-labeled ethanol by entering EMP (Fig. 33).

Metabolism of 2-C-13-Glucose to 2-C-13-Arabinitol

The two-labeled arabinitol arose by non-oxidative route through the pentose phosphate pathway (Fig. 34). A molecule of 2-C-13-fructose-6-phosphate from EMP would interact with a molecule of glyceraldehyde-3-phosphate under the influence of transaldolase. The product of such an encounter would be sedoheptulose-7-phosphate labeled in the two position. Transketolase can then transfer the top two carbons to a molecule of glyceraldehyde-3-phosphate to yield 2-C-13-xylulose-5-phosphate. Transketolase could also generate the labeled xylulose phosphate using the two-labeled fructose and glyceraldehyde as substrates. Unlabeled erythrose-4-phosphate would be the other product generated by this exchange. Isomerization and reduction of the 2-C-13-xylulose-5-phosphate yield a molecule of 2-C-13-arabinitol. Other routes through the pentose phosphate pathway may generate 2-C-13-arabinitol, but the proposed

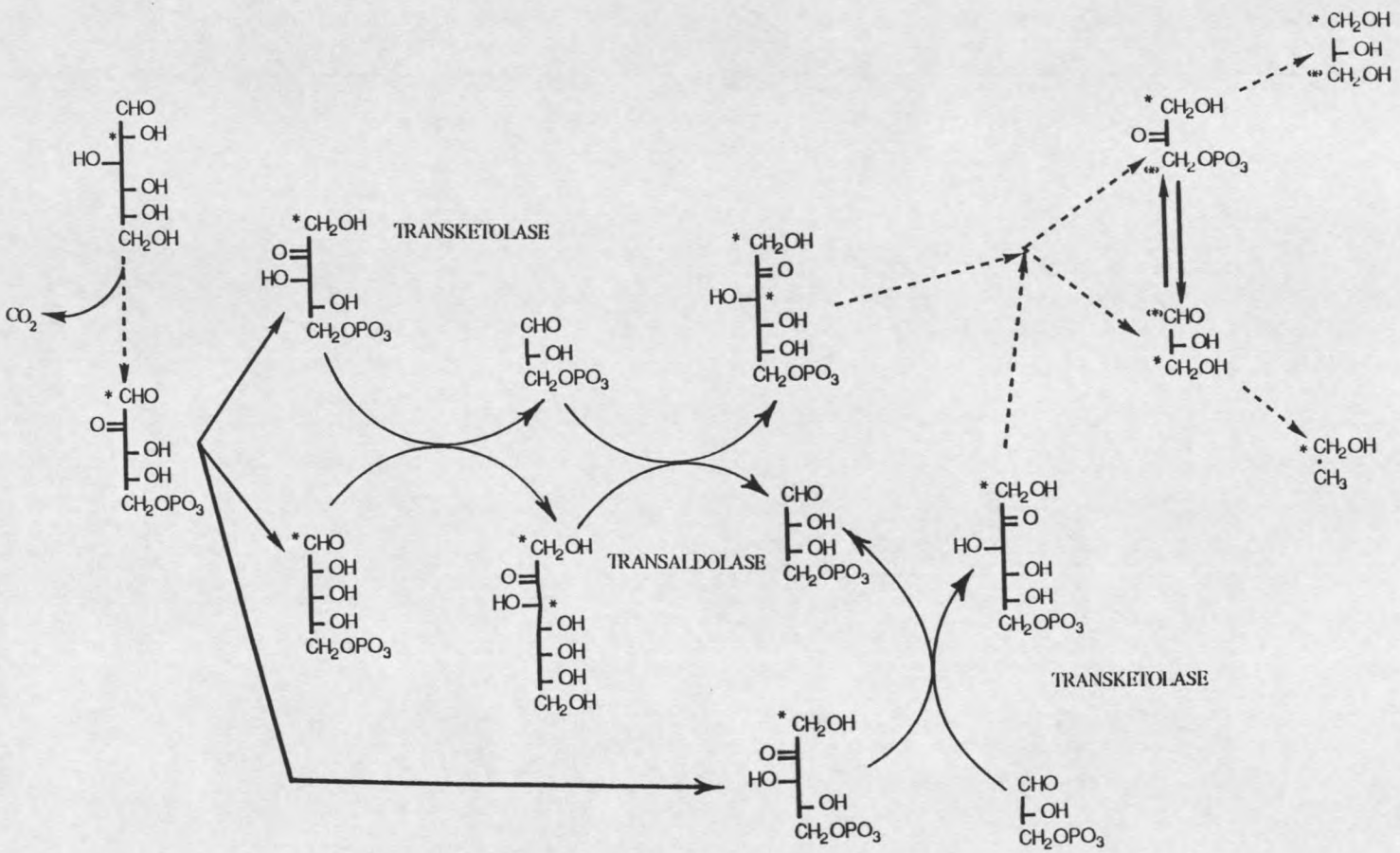

Figure 33. Pathways used by C. albicans to produce 1-C-13- and 1,3-C-13-glycerol and 2-C-13-ethanol from 2-C-13-glucose.

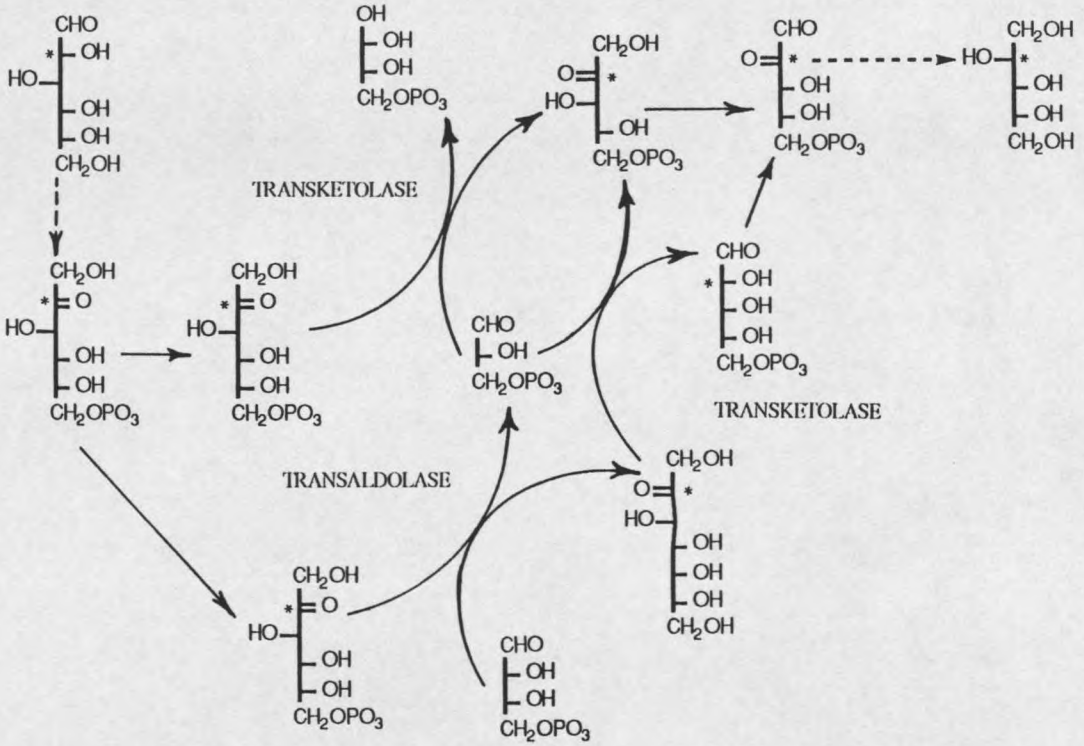

Figure 34. Pathways used by C. albicans to produce 2-C-13-arabinitol from 2-C-13-glucose.

route would predominate, therefore the other routes are ignored in this analysis.

Metabolism of 2-C-13-Glucose to 4-C-13-Arabinitol

The four-labeled arabinitol had two possible ways of being generated. Figure 35 shows that both routes count on the action of triose phosphate isomerase to convert labeled dihydroxyacetone phosphate (DHAP) into labeled glyceraldehyde. Once the glyceraldehyde is generated, it could proceed by either of two routes. The 2-C-13-glyceraldehyde-3-phosphate acts as a substrate for transketolase to make four-labeled xylulose. The other two sugars involved would be fructose-6-phosphate as the other substrate molecule and erythrose-4-phosphate as the second product. The other route again involved the actions of transketolase, except 2-C-13-glyceraldehyde-3-phosphate and sedoheptulose-7-phosphate were the reactants and 4-C-13-xylulose-5-phosphate and ribulose-5-phosphate were the products. Again, isomerization and reduction would yield 4-C-13-arabinitol.

Metabolism of 2-C-13-Glucose: Ratios of Products

The integrals of the labeled products found in Table 2 can be used to calculate the relative contributions each route had to the total amount of product produced. The 1-C-13-arabinitol constituted 50% of the total labeled arabinitol excreted by the cell, 2-C-13-arabinitol

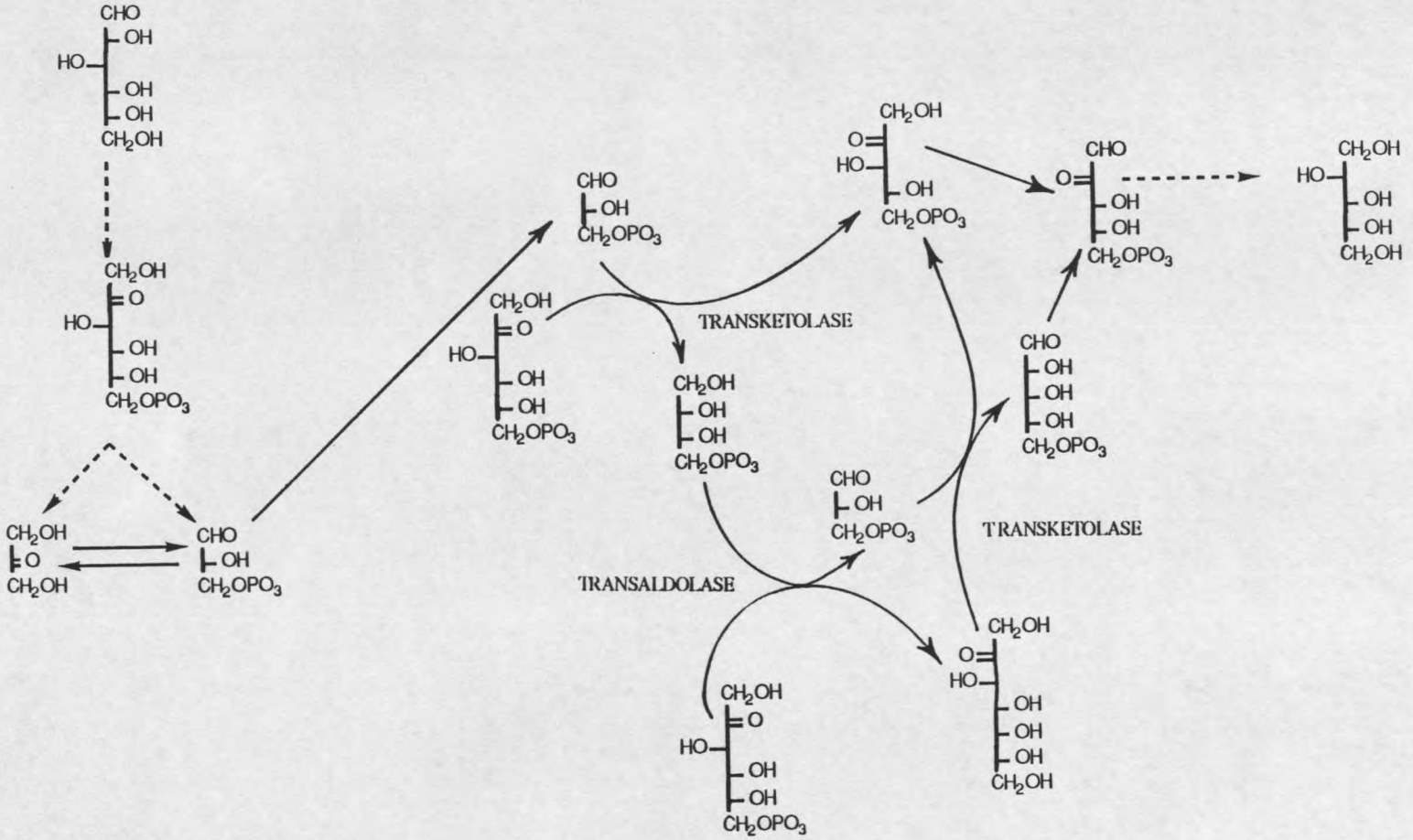

Figure 35. Pathways used by C. albicans to produce 4-C-13-arabinitol from 2-C-13-glucose.

contributed 25% and the 4-C-13-arabinitol made up the remaining 25%. These percentages reflect a diminishing contribution from the labeled glucose to the pentose phosphate pathway as the label travels down EMP. Of the 2-C-13-glucose available for conversion into pentoses, the largest fraction, 50%, comes from 2-C-13-glucose-6-phosphate. There were mainly two competing pathways for the 2-C-13-glucose-6-phosphate. The first was through EMP, and entered into the oxidative portion of the pentose phosphate pathway. The next contribution is made by 2-C-13-fructose-6-phosphate by way of the non-oxidative portion of the pentose phosphate pathway. Only half of the original amount of glucose that was converted into pentoses was available at this point. Of the original amount of glucose available 35% of it was diverted into 2-C-13-arabinitol production. There were again two paths the 2-C-13-fructose-6-phosphate could have taken. The 4-C-13-arabinitol contribution to the total arabinitol pool was 25%. Its path of contribution involved glyceraldehyde-3-phosphate, which had three competing pathways. The first was to continue down glycolysis to generate ethanol and TCA intermediates, the second was the generation of glycerol and the third was the exchange reactions used to generate 4-C-13-arabinitol. Calculations using the average integrals from Table 3 show that 80% of the glycerol pool

consisted of two-labeled glycerol and the one-labeled glycerol made up the remaining twenty percent.

Metabolism of 1-C-13-Glucose

The labeling patterns resulting from the feeding of 1-C-13-glucose reinforced the above conclusions. The 1-C-13-glucose was metabolized to arabinitol labeled in the one and the five positions. The five-labeled arabinitol is the result of label scrambling by the action of aldolase. This is no different than the mechanism suggested by the 2-C-13-glucose experiments (Fig. 36). The most plausible route for the generation of 1-C-13-arabinitol is the action of transaldolase upon 1-C-13-fructose-6-phosphate, followed by the action of transketolase upon the resulting molecule of sedoheptulose-7-phosphate or the action of transketolase upon the one-labeled fructose-6-phosphate and glyceraldehyde-3-phosphate (Fig. 37). These reactions yield 1-C-13-xylulose-5-phosphate, which upon isomerization and reduction yields 1-C-13-arabinitol. Results similar to these were obtained by Spencer et al (66). They fed C-14 labeled glucoses to an osmophilic yeast, collected the supernatant liquids just before the last of the glucose was consumed, and subjected them to degradative analyses. The cellular extracts were not analyzed. Their 1-C-14-glucose results showed the presence of arabinitol labeled in the one and the five positions. Spencer et al proposed that transketolase was responsible for the conversion of 1-C-

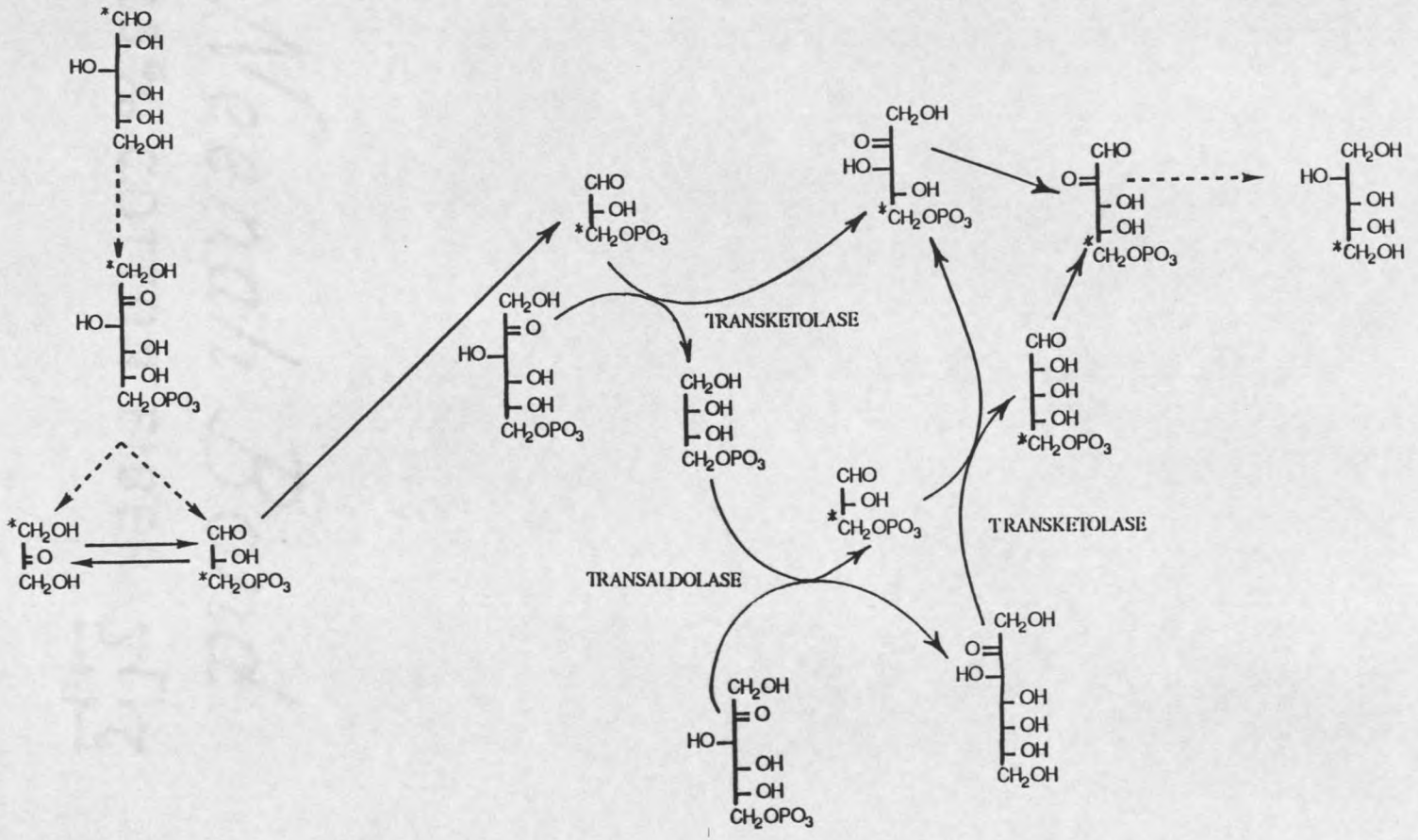

Figure 36. Pathways used by C. albicans to produce 5-C-13-arabinitol from 1-C-13-glucose.

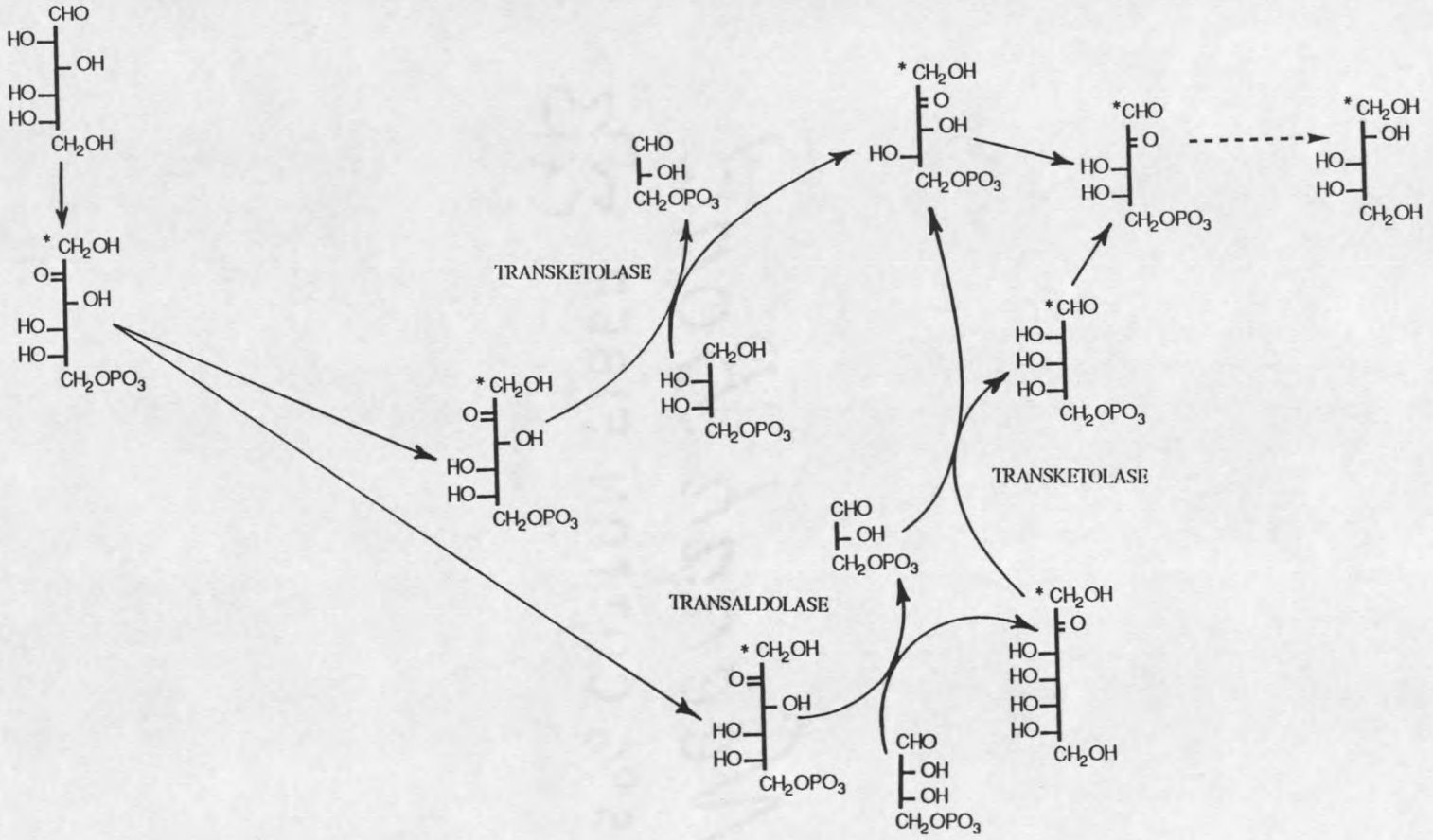

Figure 37. Pathways used by C. albicans to produce 1-C-13-arabinitol from 1-C-13-glucose.

14-glucose to one-labeled arabinitol, however transketolase alone cannot accomplish this transfer. The fact that the contributed 25% and the 4-C-13-arabinitol made up the ratios of the peak integrals of the one- to five-labeled arabinitol from the 1-C-13-glucose experiment (1.2:1.0) as compared to the two- to four-labeled arabinitol from the 2-C-13-glucose experiment (1.1:1.0) supports the conclusion that fructose-6-phosphate from EMP enters the non-oxidative portion of the pentose phosphate pathway to generate both the 1-C-13-arabinitol and the 2-C-13-arabinitol.

Metabolism of 1-C-13-Xylose

The 1-C-13-xylose experiments provide further corroboration for the pathways used to explain the results of the one- and two-labeled glucose experiments. The xylose would be metabolized first in the pentose phosphate pathway, and then it would proceed into the other pathways. The expected product of one-labeled arabinitol was produced in addition to other products. Minor amounts of two-labeled arabinitol and two-labeled glycerol were visible in the spectra, as well as a moderate amount of label resonating at 62.8 ppm (Fig. 24). The resonance at 62.8 ppm could be either glycerol or ribitol. If this peak was due to ribitol, then it would probably arise from direct isomerization and reduction. Although this is possible, such high concentrations of ribitol had not been seen previously. If the peak was due to glycerol, then the

easiest route uses the one- and three-labeled fructose-6-phosphates the would be made by the non-oxidative portion of the pentose phosphate pathway. Since one of the primary uses of the pentose phosphate pathway is to convert pentoses into hexoses that can be metabolized by EMP, these fructoses would not be an unreasonable product to expect. The labeled fructose-6-phosphate would proceed down EMP and generate labeled DHAP and glyceraldehyde-3-phosphate. The labeled DHAP could then be used to make the one-labeled glycerol. Although this is a possible way of generating the one-labeled glycerol, it has a flaw. The action of triose phosphate isomerase would convert the labeled DHAP into labeled glyceraldehyde-3-phosphate. This could then proceed down EMP, possibly generating two-labeled ethanol. Since the glyceraldehyde-3-phosphate could be labeled in either the one or the three position, only half of the ethanol generated by this pathway would have a label in its two position. There is no detectable peak at 17.1 ppm, which is where the two-position of ethanol would resonate (Fig. 24). This may only indicate that ethanol production was not of metabolic importance to the yeast, but could also indicate a lack of 1-C-13-glyceraldehyde-3-phosphate for ethanol production. This in turn implies a lack of DHAP which is essential for glycerol production. The two-labeled arabinitol arose from the three-labeled fructose re-entering the oxidative portion of the pentose phosphate

pathway. The intensity of the 2-C-13-arabinitol peak is much less than that of the 1-C-13-arabinitol peak, which would be expected because of the less direct route through which this arabinitol is generated.

Cell Extract Labeling Patterns

The pattern of labeling found in the cell extracts follows the labeling found in the supernatant liquid, with the added complication of there being little or no detectable five-labeled arabinitol in the 1-C-13-glucose experiment. This result cannot be readily explained.

Natural Abundance C-13 NMR Classification of Candida

Natural abundance can be used as a method of classifying organisms. The strains of C. albicans can be sorted based upon the ratio of trehalose to arabinitol (Table 3). An analysis of the means of the ratios of trehalose to arabinitol in the type I and type II organisms demonstrated that the two groups were distinct. Classification of a strain into one of the two groups is accomplished by calculating this ratio. If it is in the range of 0.76 - 1.53, then the strain is type I. If it is less than this ratio, than it is type II. The type III grouping is a default grouping that consisted of the single strain that did not fit into either category because of its lack of arabinitol when grown on SAAMF (Fig. 3). Although it was a limited sample, it appeared possible to identify the

several of the species of Candida and Torulopsis glabrata, as well as sort the strains of C. albicans. The spectra associated with these organisms were unique. When extended to include the spectra found in the literature, the spectral classification technique appears to be valid. The natural abundance spectra of the amoeba Acanthamoeba castellanii is dominated by resonances due to glucose, trehalose and other glucosides (28). Several resonances due probably to amino acids and fatty acids are also present. Yoshida, Murai and Moriya's spectra of plant pathogenic fungi show another pattern of resonances (43). Their spectra were dominated by mannitol and trehalose in the carbohydrate region, and the pattern of resonances from 0-55 ppm were also distinctive. Martin et al gathered spectra of the ectomycorrhizal fungi Cenococcum graniforme and Hebeloma crustuliniforme (44). Fatty acid resonances were dominate, with palmitic, oleic and linoleic acids having the highest concentrations. Including the work done with grape berries (29), frog muscle (30), nerve tissue (31), rat tissues (39), fruit pulp (41) and other studies (41-44), the use of natural abundance C-13 spectra for classification may have some utility.

Several factors may have been responsible for the lack of reproducibility found between spectra of the same strain acquired at varied times. As shown in Figure 31, each strain can have an unique spectrum. We endeavored to

minimize the environmental variations that a given strain would experience during its growth. The medium used, SAAMF, was selected because it was chemically defined and came from a commercial distributor. Since changes still occurred, other factors were probably the source. One strong possibility comes from the experiments of Dawson et al (67,68). In this work, Dawson grew the yeast Candida utilis under conditions such that 80% of the cells were in a particular phase of the cell cycle at any given time. Using such cells, he extracted and analyzed the constituents of the cells. The results demonstrated that the biochemistry of the cells vary with the portion of the cell cycle. Since natural abundance C-13 NMR observes the internal stores of the cells and if the cells harvested were not in the same portion of the cell cycle each time, then the spectra would be different.

The spectra obtained in this work demonstrated that the internal stores of compounds found in C. albicans strains were sensitive to the growth conditions of the cells. Changing the growth medium, growth temperature, or supplements to the medium resulted in changes in the spectra. This observation may prove useful. By changing one or more of these or other factors, it may be possible to induce more pronounced and reproducible changes in the cells that may more readily separate the strains based upon their carbon spectra.

SUMMARY

The study of the arabinitol metabolism and the classification of strains of Candida albicans using natural abundance and isotopically enriched C-13 NMR spectroscopy formed the core of my project. The classification problem was approached using natural abundance NMR because the spectra reported in the literature demonstrated that different organisms produced different spectra. With this background, it seemed possible to use NMR to classify the yeast. The spectra acquired during these experiments revealed a wealth of information about the cells. Finding high concentrations of arabinitol in the organisms led to a study of polyol metabolism using both natural abundance and enriched C-13 NMR spectroscopy.

Natural Abundance Spectra of C. albicans

The spectra acquired from the yeast were well resolved (5-15 Hz) and fairly intense. It proved possible to obtain spectra with acceptable signal-to-noise in a half hour experiment. The spectra had a large number of resonances, over twenty-five, and this complicated the process of assigning the peaks. However, I was able to associate over twenty of the resonances to known compounds. The most

intense resonances were due to arabinitol, glutamate, trehalose, glycerol, and alanine. A majority of the remaining resonances were due to several amino acids. These were glutamine, proline, methionine, aspartate, and aspartamine. Identification was accomplished by comparing the spectra of either compounds isolated from the cells or spectra of the intact cells to spectra of standards. The reasonableness of these identifications was verified by comparison to compounds found in yeast by other workers.

Natural Abundance NMR of Arabinitol

The response of C. albicans to media with higher and higher osmolarity was investigated. Series of growth media supplemented with salt to give a final concentration range from 0.075 to 0.600 N NaCl were used. The results indicated that all three types of C. albicans respond to the increasing salinity of the media by producing arabinitol in all salt concentrations and glycerol as the salt concentration exceeded 0.150 N. This response is similar to the responses of other halotolerant yeasts, but the salinities were not as high in my experiments.

It was also observed that the arabinitol concentration in the cells increased when the growth temperature was changed from 37°C to 28°C. This experiment had significance outside of this study in that a shift in growth temperature from 28°C to 37°C has been used to induce germination in C. albicans.

These two observations were used to maximize the production of arabinitol by the cells in the labeled substrate experiments.

Enriched Substrate NMR Experiments

The label scrambling products noted from the use of 1-C-13-, 2-C-13-glucose and 1-C-13-xylose were used to identify the pathways used by C. albicans to produce arabinitol and glycerol.

The first experiments used 2-C-13-glucose. When fed the labeled glucose, the labeled carbon appeared in the one, two and four positions of arabinitol and the one and two positions of glycerol. The one-labeled arabinitol and two-labeled glycerol would have been the only products if no label scrambling had occurred in the pentose phosphate pathway and the Embden-Meyerhoff-Parnass (EMP) pathways. The presence of one-labeled glycerol, two-labeled arabinitol and the four-labeled arabinitol indicated that the situation was more complex.

The two-labeled arabinitol resulted from one process requiring the actions of transaldolase and transketolase from the pentose phosphate pathway to produce xylulose labeled in the two position. The xylulose could then be isomerized and reduced to two-labeled arabinitol.

The four-labeled arabinitol can result from label scrambling due to the combined actions of aldolase and

triose phosphate isomerase from the EMP pathway. The two-labeled glyceraldehyde-3-phosphate generated by this process could then become the bottom three carbons of xylulose under the influences of transketolase and transaldolase. Isomerization and reduction would give 4-C-13-arabinitol.

The source of the one-labeled glycerol was the labeled fructoses that were products from the pentose phosphate pathway. These fructoses, labeled in either the one or the three positions, would be metabolized in EMP to give labeled glycerol. Label in the one or the three position give identical NMR signals.

The ratios of the integrals of the arabinitol peaks were used to calculate the contributions of the various routes to the total pool of arabinitol. The calculations showed that 50% was due 1-C-13-arabinitol, which was a product of the oxidative portion of the pentose phosphate pathway, 25% was due to 2-C-13-arabinitol, which was a product of the transfer of fructose-6-phosphate into the non-oxidative portion of the pentose phosphate pathway, and 25% was due to 4-C-13-arabinitol, which was a product of the label scrambling caused by the action of triose phosphate isomerase followed by passage through the pentose phosphate pathway. The contributions reflect the decreasing amount of the original label that could be diverted to arabinitol production.

The 1-C-13-glucose experiments were used to confirm that only one route contributed to the generation of the 2-C-13-arabinitol in the 2-C-13-glucose experiment. Since 1-C-13-arabinitol can be made by only one route from 1-C-13-glucose, and 5-C-13-arabinitol can be made by the same routes that make 4-C-13-arabinitol in the 2-C-13-glucose experiment, and if the ratio of the products in each experiment is the same, then the 2-C-13-arabinitol from the 2-C-13-glucose was made by the same route, and only that route, that made the 1-C-13-arabinitol in the $_1$-C-13-glucose experiment. This was the case. The ratios were 1.2:1.0 and 1.3:1.0, which are close enough to support this conclusion.

The 1-C-13-xylose experiments yielded 1-C-13-arabinitol, 1-C-13-glycerol as major products and 2-C-13- and 4-C-13-arabinitol and 2-C-13-glycerol as minor products.

Natural Abundance Classification

Classification of C. albicans is an important problem. A valid system of classification is essential for the tracing of the progress of an infection through a population. Identification of the strain could also be of value in treating an infection by avoiding those drugs to which that particular strain is resistant. At present there exists only a gross immunological classification system for C. albicans. It was hoped that NMR could be

successful in provided a framework for classification. Preliminary experiments showed that it was possible to identify several species of Candida and Torulopsis glabrata using the above methodology. The technique was then extended to the strain level of C. albicans. Early experiments indicated that it was possible to identify strains of C. albicans based solely on their NMR spectra. However, as more experiments were done, the spectra began to show deviations that made individual discriminations impossible. Although a classification at the individual level proved impossible, a grosser system emerged. The spectra were divided into three groups dependent upon the amount of arabinitol, trehalose, and glutamate that each had. Type I had 19 strains, type II had 5 and type III, had only one strain in it. The sole type III strain had practically non-existent levels of arabinitol and trehalose. One of the largest peaks in its spectra was an unassigned resonance at 59.4 ppm. The independence of the type I and type II strains was established by use of a t-test. The independence of the type III strain was assumed by default. The absence of arabinitol and trehalose was taken as sufficient reason to classify it as its own type.

Even though our experiments could not distinguish individual strains over a long period of time, these experiments suggest that it is possible to use natural

abundance C-13 NMR spectroscopy to classify organisms. Since different media, changing growth temperature, or the addition of supplements, such as salt, to a base medium can change the spectra of an organism, it may be possible to induce the strains to give unique spectra. Improvements in instrumentation could also prove useful. Given better instrumentation or a better understanding of the variables that cause changes in the spectra obtained, the technique may prove useful for epidemiological studies but probably not for clinical use due to the time constraints put on the experiment by the need to grow up a large number of cells.

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