Metabolism, Macromolecular Synthesis, and Nuclear Behavior of Cryptococcus albidus at 37 C

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The temperature-sensitive events which prevent Cryptococcus albidus from growing at 37 C were investigated. Cultures incubated at 37 C immediately after inoculation did not increase in optical density nor in cell numbers, and by 24 h 90% of cells in such cultures were deformed and dead. When cultures in log phase were shifted from 23 to 37 C the optical density increased but the cell numbers did not. Morphological observations revealed that the increase in turbidity at 37 C represented enlargement and distortion of cells without appreciable replication. Uptake and incorporation of $^{14}$C-leucine were similar at 23 and 37 C. There was no difference in $^{14}$CO$_2$ evolution from cells at either temperature. Uptake and incorporation of adenine-$8^{-14}$C into RNA was slightly lower in cells incubated at 37 C. There was, however, a 60% reduction in incorporation of adenine-$8^{-14}$C into DNA after 3 hr at 37 C. Nuclear staining revealed that nuclear migration did not occur in cells incubated at 37 C. Thus the data indicate that both adenine incorporation into DNA and nuclear migration prior to nuclear division by C. albidus are temperature sensitive.

The ability to grow at 37 C is one of the criteria used to distinguish various species in the genus Cryptococcus. Strains of C. albidus generally do not grow at 37 C, but many remain viable at that temperature for some time. Metabolism, macromolecular synthesis, and nuclear behavior of this yeast at 23 and 37 C were studied in an attempt to identify the temperature-sensitive step(s) which precludes growth at the higher temperature.

MATERIALS AND METHODS

Organism. The strain of C. albidus used in this study (no. 378, stock culture collection of this Department) was grown on slants of glucose-peptone-agar (2) at room temperature for 3 days and subsequently refrigerated. Subcultures were made at monthly intervals. The cells were grown and prepared for the experiments as described previously (12).

Growth of C. albidus. The growth of the fungus was measured by 3 methods: (i) nephelometry, (ii) direct hemocytometer counts, and (iii) quantitative plate counts.

Nephelo triple-baffled culture flasks (Belloco, Vineland, N.J.), each containing 20 ml of glucose-peptone-broth, were used in those experiments in which growth was measured by increases in turbidity. The flasks were inoculated with 0.4 ml of a 24-h broth culture and incubated at 23 or 37 C on a rotary-action shaker (model "V", New Brunswick Scientific Co., New Brunswick, N.J.). At determined intervals, the turbidity of the broth was measured with a Klett-Summerson photoelectric colorimeter (Klett Mfg. Co., Inc., New York, N.Y.). Viability of the cells was determined by eosin-y dye exclusion (13).

Direct counts were made on cultures prepared and inoculated as in the preceding paragraph. A 1-ml sample was withdrawn at determined intervals and appropriately diluted. The cell numbers were then counted in a hemocytometer.

The inoculum for quantitative plate counts was grown in 30 ml of glucose-peptone-broth for 24-h at 23 C. The cells were harvested, washed, and suspended in 10 ml of 0.15 M NaCl solution. Glucose-peptone-broth (100 ml) was inoculated with 1 ml of the saline suspension. At determined intervals, 1 ml was withdrawn and serially diluted in saline. A 0.1-ml sample of the final dilution was plated on glucose-peptone-agar plate in triplicate. The colonies were counted after 3 days of incubation at 23 C.

Morphological development of individual cells. Twenty milliliters of glucose-peptone-broth was inoculated with 0.4 ml of a 24-h culture and incubated at 23 C for 12 h. One loopful of cell culture was then transferred onto a glucose-peptone-agar plate which had been prewarmed at 37 C. The plate was mounted on the stage of a Leitz light microscope, and the cells were photographed at 0 time and 6 h after shifting to 37 C.

Nuclear staining. Nuclear division was studied in material prepared by a combination and a modification of the procedures used by Hartwell (6) and by
Esposito et al. (3). A 1-ml sample from a 20-ml cell culture incubated at 23 or 37 C was removed at determined intervals and added to 9 ml of 0.15 M NaCl solution containing 3.7% Formalin. After fixation in NaCl-Formalin (samples could be left for as long as 20 h before workup), cells were collected by centrifugation and affixed to cover slips which had a thin coating of 5% egg white. The yeast-coated cover slips were immersed in 70% ethanol for 5 min, incubated in 1% NaCl solution at 60 C for 45 min, and treated with ribonuclease (0.2 mg of pancreatic ribonuclease per ml of 0.05 M tri(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.4) at 60 C for at least 60 min. The cover slips were rinsed once with water and once with 0.02 M phosphate buffer (pH 6), and were incubated for 3 h in 10 ml of 0.02 M phosphate buffer (pH 6) containing 0.3 ml of a Giemsa stain solution (Allied Chemical, Morristown, N. J.). The Giemsa stain-buffer was made fresh on the day of staining. The preparations were destained for 30 s in 95% ethanol, dehydrated for 10 min in xylene, and mounted in Permount (Fischer Scientific Co., Fairlawn, N.J.) on slides.

Measurement of l-leucine uptake and incorporation. Uniformly labeled l-leucine-14C with a specific activity of 331 mCi/mmol (Amersham/Searle Corp., Des Plaines, Ill.) was used. The radioactive amino acid was diluted with unlabeled l-leucine (Calbiochem, Los Angeles, Calif.) to achieve the desired molar concentrations. The uptake and incorporation of the amino acid were measured as described previously (12) in Littman's thiamine-base salts medium (10).

Collection and measurement of CO2 derived from l-glutamic acid. The production by C. albidus of 14CO2 from uniformly labeled glutamic acid was measured as described previously (12).

Ribonucleic and deoxyribonucleic acid synthesis. The synthesis of ribonucleic acid (RNA) and of deoxyribonucleic acid (DNA) was monitored by the incorporation of adenine-8-14C. Cells were grown in Littman's synthetic medium (10) at 23 C for 22 h. The cells were harvested, washed, and adjusted to 0.4 mg (dry weight) per ml of the synthetic medium. Equal volumes of 1.31 x 10-3 M 14C adenine (adenine-8-14C·HCl, specific activity of 6.83 mCi/mmol, New England Nuclear Corp., Boston, Mass.) and 0.4 mg of cell suspension per ml were placed in separate flasks in a water bath shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 23 or 37 C for 30 min. After temperature equilibration, the adenine solution was added to the cell suspension in synthetic medium, and the flask was shaken throughout the experiment. The uptake of 14C adenine was measured by filtering 1.0-ml samples at determined intervals and measuring the radioactivity of the filtrate. In keeping with the experience of others (11), the more conventional precursor, tritiated thymidine, could not be used in studies on DNA synthesis, because it was not taken up by C. albidus.

The synthesis of RNA was measured by a modification of the procedure of Hartwell (5). A 5-ml sample of the 14C adenine supplemented medium was withdrawn at appropriate intervals, mixed with 5 ml of cold 10% trichloroacetic acid, incubated for 30 min, and centrifuged at 2,600 x g for 10 min. The pellet was resuspended in 5 ml of 1 N NaOH, incubated at 23 C overnight, and then cooled to 4 C. Five ml of 20% trichloroacetic acid was added to the resuspended material. The mixture was incubated for 30 min and centrifuged at 2,600 x g for 10 min. The supernatant fluid was retained, the pellet was washed with 5 ml of cold 5% trichloroacetic acid by centrifugation, and this wash was combined with the first. The supernatant fluid, which contained the NaOH-digested, cold, trichloroacetic acid-soluble RNA fraction, was analyzed for radioactivity.

The procedure of Hartwell (6) was followed in the measurement of DNA synthesis. Briefly, a 1-ml sample was removed at determined intervals and added to 1 ml of 2 N NaOH. After incubation at 23 C overnight, the samples were chilled, and 100 μg of carrier DNA (salf thymus DNA, Worthington Biochemical Corp., Freehold, N.J.) and 1 ml of 50% trichloroacetic acid were added. The samples were then filtered and washed 10 times with 1 ml of cold 5% trichloroacetic acid. The pads were dried and analyzed for radioactivity.

Measurement of radioactivity. Radioactivity was measured by counting in a liquid scintillation counter in a manner described previously (12).

RESULTS

Growth and morphology of C. albidus at two different temperatures of incubation. C. albidus, incubated at 23 C, had a generation time of 2 h after a 2-h lag (Fig. 1). Cells incubated at 37 C did not increase in number, and those shifted to 37 C after a period of growth at 23 C (6 h) stopped dividing. The data in Fig. 1 were obtained from determinations made by direct hemocytometer counts, but the same results were obtained from experiments in which growth was assessed by quantitative plate counts (data not shown). When experiments were performed by measuring growth by nephelometry, some increases in turbidity were noted after cultures in log phase were shifted from 23 to 37 C (Fig. 1). Microscope examination of cells from cultures shifted to the nonpermissive temperature revealed that most of them were in an arrested process of budding. Morphological alterations were observed in the cells by 4 h after the shift to 37 C (Fig. 2). In several preparations, cells with more than one bud were seen. Thus, the increase in turbidity was due to increase in cell size and to appearance of distorted forms and not to increases in cell numbers.

After 18 h of incubation at 37 C, 90% of the cells were still viable, but many were deformed. However, by 24 h of incubation at 37 C, nearly all of the cells were dead (eosin-y dye exclusion test; 13) and deformed. The appearance of the cells indicated that a disturbance in some step
of mitosis or of cell separation might be involved in growth inhibition at 37 C.

**Transport of amino acids by C. albidos.**
Previous work from our laboratory showed that glutamic acid uptake by C. albidos was severely inhibited at 37 C (12). Data from a comparative study of the uptake of glutamic acid and of leucine by the fungus are shown in Fig. 3. It can be seen that, in contrast to the results with glutamic acid, the rate of uptake of leucine was approximately the same at 23 and at 37 C. The optimal temperature for transport of leucine was 30 C, whereas that for transport of glutamic acid was 28 C (Fig. 3).

The kinetics of leucine transport by C. albidos were not examined as closely as were those of glutamic acid transport (12). The leucine concentration employed (Fig. 3) was chosen arbitrarily to correspond to that at which a high-affinity active-transport system was known to be operative for glutamic acid (12). The substrate concentration used (Fig. 4) was chosen for convenience.

**Macromolecular synthesis and metabolic activities of C. albidos at permissive and nonpermissive temperatures.** The uptake and incorporation of L-leucine-14C by cells of C. albidos were not affected by the restrictive growth temperature of 37 C. L-Leucine uptake began immediately after the addition of the amino acid to the cell suspension. The cells at both 23 and 37 C accumulated the amino acid as a linear function of time. Sixty percent of the

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**Fig. 1.** The growth of C. albidos at two temperatures of incubation in glucose-peptone broth. Data are from direct hemocytometer counts at indicated time intervals. Symbols: O, incubated at 37 C; Δ, incubated at 23 C; ▲ incubated at 23 C for 6 h, then shifted to 37 C.

**Fig. 2.** Morphology of yeast cells of C. albidos grown in glucose-peptone-broth. Cells incubated at 23 C for 12 h (A), at 37 C for 12 h (B), and at 37 C for 18 h (C). Magnification is x640.
amino acid was accumulated by the cells incubated at 23 °C after 30 min. The rate thereafter decreased, and at the end of the 4-h experimental period, approximately 80% of the amino acid had been accumulated by the cells. Cells incubated at 37 °C accumulated approximately 70% of the amino acid from the medium within 1 h; thereafter, no further accumulation was observed. The accumulated amino acid was incorporated into the cold, trichloroacetic acid-insoluble fraction without any lag. The initial rate of incorporation was almost the same at both temperatures. The rate at 23 °C began to decrease after 30 min, whereas that at 37 °C did so after 15 min. At the end of the experiment, 19% of the 14C activity taken up at 37 °C and 23% of that taken up at 23 °C could not be accounted for. It is likely that a part of the accumulated L-leucine was metabolized and lost as CO2 (4). From these data it was concluded that incorporation of amino acids into trichloroacetic acid-insoluble materials was not inhibited at 37 °C.

C. albidus metabolizes glutamic acid, and about 25% of the total uptake is evolved as 14CO2 (12). Cells incubated in glutamic acid at 23 °C and then shifted to 37 °C continued to evolve CO2 at the elevated temperature (Fig. 5). Therefore, the metabolism of glutamic acid was unimpeded at a temperature at which it was not transported.

The uptake and incorporation of 14C-adenine into RNA were not substantially different at 37 and 23 °C (Fig. 6). Adenine-6-14C was accumulated by the yeast cells at 23 and 37 °C as a linear function of time, after a lag of about 30 min. By 2.5-h at 23 °C the yeasts had accumulated 90.4% of the total radioactivity in the medium, and after the same period of time at 37 °C, the cells had accumulated 82.4% of the total radioactivity. The accumulated adenine was incorporated into the RNA fraction. By 2.5-h the rate of incorporation at 23 °C began to decrease and continued at the slower rate up to 6 h, at which time 85.3% of the total radioactivity accumula-

**Fig. 3.** The influence of temperature on the rate of uptake of L-glutamic acid and L-leucine by C. albidus. The system consisted of 0.2 mg (dry weight) of cells per ml and 1 × 10⁻⁴ M L-amino acid in Littman's thiamine-basal salts medium (12). The rate of uptake per hour per milligram (dry weight) of cells was computed from observations made at 60 min for L-glutamic acid (to allow for an initial lag of 30 min previously noted (12) under these conditions) and from observations made at 30 min for L-leucine (no lag observed). Both observations were made at a time during which the curve was linear.

**Fig. 4.** Time course of uptake and incorporation of L-leucine by C. albidus at 23 and at 37 °C. The system consisted of 0.2 mg (dry weight) of cells per ml and 1 × 10⁻⁴ M L-leucine in Littman's thiamine-basal salts medium. (See Materials and Methods.) Symbols: ○, total uptake; Δ, cold, trichloroacetic acid-insoluble fraction; and O, cold, trichloroacetic acid-soluble fraction.
controls after 3 h at 37°C (Fig. 7). The inability of the cells to incorporate adenine into DNA may have occurred through damage to the proteins involved in DNA synthesis or through damage to the DNA itself.

**Nuclear division of C. albidus.** After incubation at 23 and 37°C for 4 h in glucose-peptone medium, the yeast cells were fixed and stained to reveal nuclei (Fig. 8). The nuclei of the cells incubated at 23°C were readily distinguishable. Examination of about 750 cells from an asynchronously dividing culture revealed that 5% were in nuclear division as evidenced by the presence of the nucleus in the isthmus separating parent cell and bud. Approximately 30% of the cells at this temperature had buds of various sizes. In many cases, the nucleus was an elongated structure extending into both cells. These results were similar to those reported by Hartwell et al. (9) with *Saccharomyces cerevisiae*.

Some difficulty was encountered in readily distinguishing the nuclei of cells incubated at 37°C when the staining procedure used was exactly the same as that used on cells incubated at 23°C. Increasing the period of digestion with ribonuclease (RNase) to 2 h resulted in distortion of cells. Shorter periods of Giemsa staining or longer destaining did not improve the results. However, the nuclei became clearly distinguishable and the cells were not distorted when the preparations were incubated at 60°C in 1 N HCl for 13 min prior to staining and after the 1-h period of RNase digestion. Nuclei of cells incubated at 37°C and prepared in this manner appeared as a compact, intensely staining mass within the parent cells. Approximately 30% of the yeasts had buds, but none of these buds were nucleated. This result shows that nuclear

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**Fig. 5.** Evolution of 14CO2 from L-glutamic acid-14C (U) (1 x 10^-4 M) by C. albidus after temperature shift from 23°C (0 time) to 37°C (4 h). Time of shift indicated by arrow.

**Fig. 6.** Uptake and incorporation of 14C-adenine into RNA by C. albidus incubated at 23 and at 37°C. Symbols: - - , total amount of radioactivity in the medium; O, total uptake; O, incorporation into RNA (see Materials and Methods).

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**Fig. 7.** Uptake and incorporation of 14C-adenine into DNA by C. albidus incubated at 23 and 37°C. Graph A: total amount of radioactivity in medium (-); uptake 23°C (O); and uptake at 37°C (O). Graph B: incorporation into DNA at 23°C (O) and 37°C (O). Note that the values on the ordinate of graph B are 10-fold less than those in graph A.
migration and division did not occur at the restrictive temperature. The cells incubated at 37 C were bigger than those incubated at 23 C (Fig. 8).

Thus, when cells of C. albidus are incubated at 37 C, growth ceases, the overall metabolism of the cells continues, and leucine is incorporated into proteins and adenine into RNA. But nuclear migration is inhibited and DNA synthesis is severely compromised.

**DISCUSSION**

The cell division cycle of S. cerevisiae begins with the initiation of bud formation, followed rapidly by DNA replication which is completed within the first 25% of the time required for the entire cell cycle (9). Nuclear division, which occurs approximately three-fourths of the way through the cycle (1), is followed by cell separation. The bud enlarges throughout the cycle until it reaches a size approximately the same as that of the parent cell at the time of cell separation.

Hartwell (9) studied the morphological development of temperature-sensitive mutants of S. cerevisiae after a shift to a restrictive temperature and defined the time in the cell cycle at which the temperature-sensitive function would normally be performed as the "execution point." Cells that had just passed this point at the permissive temperature would progress through one more cycle of division. The stage at which the cells collected when the sensitive function was not performed was defined as the "termination point."

Some similarities between the observations on temperature-sensitive mutants of S. cerevisiae and ours with C. albidus are apparent. Cells of C. albidus enlarged after temperature shift and became peanut-shaped cells or distorted cells (Fig. 2). Nuclear staining studies showed that the nuclei did not migrate to the isthmus between the buds and parent cells (Fig. 8). Therefore, C. albidus terminated development at an early stage of nuclear division, namely, nuclear migration. After the block in nuclear division at the restrictive temperature, cell separation and additional DNA synthesis did not take place. This was to be expected, because cell separation seems to depend on successful completion of nuclear division (7). Because cell separation and DNA replication are not prerequisites for bud initiation (7, 8, 9), multiple budding was seen in some of our preparations incubated at 37 C.

Hartwell and Culotti studied the morphological development of each individual cell by time lapse photomicroscopy at a restrictive temperature and related a cell’s position in the cell cycle at the time of the temperature shift (determined by bud size) to its development at the restrictive temperature (one cell with a large bud or two cells each with a large bud) (1). The time or position in the cell cycle (expressed in fractions of a cell cycle) was recorded as the length of the bud divided by the length of the parent cell. The execution point of all the temperature-sensitive mutants which were defective in nuclear division was from the very beginning of the cell cycle (0.02) to approximately one-half way through the cell cycle (0.45) (1). All of the numbers which represented

![Fig. 8. The appearance of nuclei of cells of C. albidus incubated at 23 C for 4 h (A), at 37 C for 4 h (B), and at 37 C for 8 h (C). Note chromatin in the isthmus between parent cell and bud (arrow). Magnification is ×1,800.](http://jb.asm.org/.../onOctober26,2017byguest)
fractions of a cell cycle were significantly before the point at which nuclear division occurred (approximately three-fourths of the way through the cell cycle). We performed a similar study on the morphological development of individual cells of *C. albicans* on agar plates after a shift to the restrictive temperature. As shown in Fig. 9, those cells with a bud size (i.e., the length of the bud divided by the length of parent cell) of 0.2 to 0.4 became one cell with one large bud. Those cells with a bud size greater than 0.4 to 0.6 became two cells with two buds. The execution point for *C. albicans* thus was computed to be at a bud size of approximately 0.4 that of the parent cell.

Cells incubated in the thiamine-basal salts medium which was used for the study of L-leucine uptake and incorporation did not replicate during the period of the experiments, but the generation time of *C. albicans* in Littman's synthetic medium was 2.8 h, and this interval was taken into account in the study of adenine uptake and incorporation. The cells accumulated about the same amount of adenine into the RNA fraction when incubated at 23°C as they did when incubated at 37°C. Because the cells incubated in synthetic medium at 37°C did not divide, the amount of RNA in individual cells would have been nearly twice that in individual cells incubated at 23°C. This excess RNA could account, in part, for the early difficulty encountered in efforts to selectively stain the nuclei of cells incubated at 37°C.

Although protein and RNA synthesis in *C. albicans* was not affected by incubation at 37°C, incorporation of radioactivity into the DNA fraction was markedly inhibited. However, the cells did incorporate some adenine into the NaOH-resistant, trichloroacetic acid-insoluble precipitate. Because many events occur preparatory to nuclear division, any one of these processes could be temperature-sensitive (7) and, in turn, those genes that control these processes could be defective. DNA replication, for example, is a prerequisite for the completion of nuclear division, and any subtle abnormalities in DNA replication, which might not be detected when net DNA synthesis is monitored, would result in a block in nuclear division.

The uptake of L-glutamic acid was severely inhibited at 37°C, but that of L-leucine was not, indicating that there is some difference in the transport site for these two amino acids. The uptake of glutamic acid has been thoroughly examined (12). The evidence is that glutamate was taken up by two active transport systems. Preliminary studies revealed that a similar situation exists with leucine.

The initial observations on the growth of *C. albicans* at the two temperatures of incubation revealed a difference in behavior between stationary-phase cells and log-phase cells shifted to 37°C. Thus, cells inoculated into media and incubated immediately at 37°C did not grow. Their failure to do so could be a reflection of their inability to emerge from stationary-phase growth. In contrast, cells in log-phase growth (i.e., incubated at 23°C for 6 h) and shifted to 37°C at
C failed to continue through the cell cycle. These two effects of temperature on growth need not be related to the same temperature-sensitive gene. Additional research is necessary to solve this point.

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**LITERATURE CITED**