Effect of Temperature on Saprophytic Cryptococci: Observations Relating to Wall Biosynthesis at Non-permissive Growth Temperatures

By R. DABBAGH*, N. F. CONANT AND R. O. BURNS†

*Department of Microbiology and Immunology,
Duke University School of Medicine,
Durham, North Carolina 27710, U.S.A.*

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SUMMARY

An attempt was made to define the basis of temperature-induced aberrant wall biosynthesis in *Cryptococcus diffluens*. A quantitative determination of the sulphhydryl content of the cell surface of *C. diffluens* as an index of a protein disulphide reductase activity failed to reveal differences between cells cultured at permissive and non-permissive growth temperatures. Assays of β -1,3-exo-glucanase showed no abnormal activities which could be responsible for the temperature-induced aberrations. *Cryptococcus diffluens* excreted low molecular weight compounds non-specifically; UMP and AMP were the predominant nucleotides excreted. Uridine diphosphoglucose, a precursor of glucan biosynthesis, was accumulated and excreted by *C. diffluens* at non-permissive but not at permissive growth temperatures. A correlation exists between the rate of yeast multiplication at permissive temperatures and the extent of residual growth upon subsequent exposure to a non-permissive temperature.

INTRODUCTION

Dabbagh, Conant, Nielsen & Burns (1974) presented evidence suggesting that the inability of the saprophytic cryptococci to grow at temperatures above 37 °C is because of lysis induced by defective wall biosynthesis. A tentative conclusion was made that the site of temperature-induced aberrant wall biosynthesis is at the budding site. This conclusion was based on observation of sucrose-stabilized *Cryptococcus diffluens* at 37 °C as well as the normal budding process where cells contain single buds; the aberration seen in the stabilized cells is a single pore in budless cells. These features, together with the observation that the point of most active wall biosynthesis in other budding yeasts is at the budding site (bud tip) (Johnson & Gibson, 1966), support this conclusion.

At least two types of degradative enzymes have been conjectured to be involved in the initiation of bud formation in yeast. One of these, protein disulphide reductase, has been implicated in the budding process in *Candida albicans*. Nickerson & Falcone (1956) showed that a permanently filamentous mutant of this dimorphic fungus did not possess the reductase whereas the normal yeast form did. A model was proposed whereby 'softening' of the cell wall by the action of the reductase initiates bud formation. The other type implicated in bud initiation and wall biosynthesis is β 1,3-glucanase. Matile, Cortat,

* Present address: Department of Microbiology, College of Medicine, University of Baghdad, Baghdad, Republic of Iraq.

† To whom reprint requests should be sent.

Wiemken & Frey-Wyssling (1971) have demonstrated that much of the glucanase present in exponentially growing cells of *Saccharomyces cerevisiae* is contained in vesicles which are derived from the endoplasmic reticulum. Electron micrographs of budding cells show that these vesicles are clustered at the apex of the growing bud. It is known that bud extension occurs by apical wall synthesis (Johnson & Gibson, 1966). It is also known that the level of glucanase-containing particles is much lower in stationary-phase yeast cells (Matile *et al.* 1971). Collectively, these results strongly implicate glucanase in yeast wall biosynthesis. The vesicles described above are believed to provide a mechanism whereby the yeast can transport glucanase to the site of active wall biosynthesis. It is thought that these vesicles discharge glucanase into the area of wall biosynthesis; the action of the glucanase thus provides primer ends upon which glucan extension takes place. Excessive activity at 37 °C of either reductase or glucanase could explain the observation on saprophytic cryptococci.

Perturbation of wall biosynthesis is frequently accompanied, in bacteria at least, by the accumulation of pertinent nucleotide wall precursors. The extension of the glucan component of yeast walls is thought to occur by the action of UDP glucose-glucosyl transferase (Johnson, 1968). Although this enzyme has not been reported in the cryptococci, the action of 2-deoxyglucose on wall biosynthesis in these organisms, coupled with the action reported for this analogue in other yeasts, supports the expected role of this enzyme in wall biosynthesis in the cryptococci.

This paper includes the results of indirect and direct analyses of the reductase and glucanase, as well as qualitative analysis of culture filtrates and cell extracts derived from *C. diffluens* grown at permissive and non-permissive temperatures.

METHODS

Cultivation of organisms. Cultures were grown as described by Dabbagh *et al.* (1974).

Radioactivity measurements. Radioactively labelled cells were collected on pre-wetted Whatman GF/C filter discs. The discs were washed 4 or 5 times with distilled water, then with absolute ethanol, dried under an infra-red lamp, and placed in scintillation vials containing 15 ml of scintillation fluid containing 15.2 g 2,5-diphenyloxazole and 378 mg 1,4-bis-[2(5-phenyloxazolyl)]-benzene in 4.5 l toluene. The samples were counted in a Beckman model LS-233 liquid scintillation spectrometer.

Removal of capsule. Cells of *C. diffluens* grown at 27 °C in minimal medium were treated with an equal volume of dimethylsulphoxide by stirring at room temperature for 20 min, removed by centrifugation, washed once with 0.85 % saline and then resuspended in saline. Indian ink preparations of cells treated in this manner showed no visible capsule.

Preparation of cell extracts. Extracts were prepared by sonication with a Branson 110 W sonifier; the power setting of the instrument was adjusted to the size of the sample treated. Cell breakage was monitored by direct microscopic examination and was usually greater than 80 %. Extracts were also prepared by means of a Mickle disintegrator. Approximately 0.5 g yeast and 20 g ballotini glass beads were suspended in 10 ml of 0.01 M-potassium phosphate pH 5.5; the mixture was placed in the Mickle apparatus and subjected to vibration for 20 min. Cell debris and glass beads were removed by centrifugation.

β -1, 3-Glucanase assay. β -1,3-Glucanase activity was measured according to the method of Brock (1965). One ml of either cell suspension or extract was added to 1 ml of 0.0066 M-*p*-nitrophenyl- β -D-glucoside at 30 °C. At the desired time 0.5 ml of the incubation mixture was removed and added to 4.5 ml of 4 % sodium carbonate. The absorbance of the yellow

colour produced by the release of *p*-nitrophenol was determined at 430 nm in a Bausch and Lomb Spectronic 20 colorimeter.

Quantification of UDP-glucose by means of UDP-glucose dehydrogenase. Dehydrogenase solutions were prepared by dissolving 3 mg of a partially purified preparation of this enzyme in 1 ml of 0.01 M-potassium phosphate, pH 7.5. A typical reaction mixture contained: NAD, 0.0125 μ mol; sodium glycinate pH 8.5, 100 μ mol; UDP-glucose dehydrogenase, 0.15 mg; the sample to be analysed; and water to make 1 ml. Two controls were run with each determination; one lacked NAD and the other the dehydrogenase. The course of the reaction was followed to completion by monitoring the formation of NADH at 340 nm with a Beckman DU spectrophotometer equipped with a Gilford model 2000 multiple-sample absorbance recorder. The molar concentration of UDP-glucose is equal to half the molar amount of NADH produced. NADH was quantified by using an extinction coefficient of 6.22×10^3 (Kornberg & Horecker, 1953).

Ultraviolet absorption spectra. These were obtained with a Beckman Acta V recording spectrophotometer.

Separation of nucleotides. Nucleotide-containing samples were adjusted to pH 5.0 with N-acetic acid. These samples were then added to columns which were constructed by layering 50 mg of charcoal (Norite A) between two layers of Celite. The columns were then washed 3 or 4 times with water. The nucleotides were eluted with 50 % ethanol adjusted to pH 11 with ammonium hydroxide.

Paper chromatography. Three solvent systems were used: (i) isobutyric acid-ammonium hydroxide-water, 6:1:33; (ii) ethanol-ammonium acetate - 77 g ammonium acetate were dissolved in 750 ml water, adjusted to pH 7.5 with ammonium hydroxide, diluted to 1 l, and 300 ml of this solution were mixed with 700 ml ethanol; (iii) 100 ml of 0.01 M-potassium phosphate pH 6.8, 60 g ammonium sulphate and 2 ml N-propanol. Descending chromatography was used with Whatman No. 1 filter paper. The chromatograms were dried and u.v.-absorbing spots located with a UVS-12 mineral light (Ultraviolet Products Inc., San Gabriel, California, U.S.A.).

Reagents and chemicals. [14 C]*N*-ethylmaleimide was obtained from Schwartz Bioresearch Inc., 1,5-difluoro-2,4-dinitrobenzene from Pierce Chemical Co., 2-mercaptoethanol from Chemical Fabrik, Switzerland and uridine, uridine-5'-diphosphoglucose, uridine diphosphoglucose, uridine diphosphoglucose dehydrogenase, *p*-nitrophenyl- β -D-glucoside and thymidine from Sigma. Diphosphopyridine nucleotide, and the sodium salts of UTP, UDP, UMP, AMP, ADP and ATP were from P-L Biochemicals Inc. All other chemicals were reagent grade.

RESULTS

Hyperactivity of disulphide reductase should be accompanied by an increase in the sulphydryl content of the walls of *C. diffluens* at 37 °C, and comparative measurements of surface sulphydryl groups in whole cells obtained from 27 and 37 °C should show this difference. *N*-ethylmaleimide, an alkylating agent, was used to determine the relative abundance of sulphydryl groups on cells of *C. diffluens* (Riordan & Vallee, 1967). Samples (0.5 and 1.0 ml) were removed from a culture of *C. diffluens* growing at 27 °C and immediately pipetted into 1 ml of [14 C]*N*-ethylmaleimide (0.003 M, specific activity 0.3 μ Ci/ μ mol). The culture was then shifted to 37 °C and samples were removed at 4, 8, 12 and 24 h and added to the alkylating agent. The culture-alkylating reagent mixture was always incubated at room temperature for 30 min. The reaction was stopped by adding 1 ml of 0.05 M- β -mercaptoethanol to each tube. This procedure effectively removes the excess

Table 1. Labelling of *C. diffluens* with [^{14}C]N-ethylmaleimide

Sampling time* (h)	Counts/min	
	0.5 ml sample	1.0 ml sample
0	988	1655
4	972	1700
8	741	1562
12	922	1249
24	892	1034

* Measured from the time the culture was shifted to 37 °C.

Table 2. Labelling of capsulated and decapsulated cells of *C. diffluens* with [^{14}C]N-ethylmaleimide

Sampling time* (h)	Counts/min			
	Control		Decapsulated	
	0.5 ml sample	1.0 ml sample	0.5 ml sample	1.0 ml sample
0	1406	1884	1329	1771
8	1234	1731	1201	1685

* Measured from the time the culture was shifted to 37 °C.

alkylating reagent. The samples were filtered and counted for radioactivity. The results of this experiment are shown in Table 1. It is evident that no increase in alkylation of the cells occurred during the course of incubation at 37 °C. Since *C. diffluens* possesses a capsule which might impede the penetration of the alkylating agent to the wall, a control experiment was performed and the alkylation of cells with and without capsules was compared. Encapsulated and decapsulated cells (see Methods) were labelled with [^{14}C]N-ethylmaleimide as described above. The results of this experiment, which are shown in Table 2, illustrate that the degree of labelling was essentially the same in both types and indicate that the capsules exerted no effect on the level of alkylation of the cells.

The experiment described above might fail to reveal any local enrichment of sulphhydryl content on the cell surface because of differences masked by the high background provided by the remaining surface of the organism. To explore the possibility of local enrichment of sulphhydryl groups the bifunctional sulphhydryl reagent 1,5-difluoro-2,4-dinitrophenol was used. Treated cells whose surfaces were locally enriched for sulphhydryl groups would be expected to show occasional grouping in the presence of this reagent. Cells obtained from a 37 °C sucrose-stabilized culture, and either treated with dimethylsulphoxide or left untreated, were concentrated to about 10^9 cells/ml and reacted with the sulphhydryl reagent. Microscopic examination failed to reveal any cell coupling and therefore no evidence exists for local concentrations of sulphhydryl groups.

The assay method for β -1,3-glucanase was more direct. Glucanase assays were performed on whole cells, cell extracts, and culture filtrates which were obtained from cultures of *C. diffluens* grown at 27 and 37 °C. Cells were prepared from 12 and 24 h 27 °C cultures, or from sucrose-stabilized cultures which had been incubated for 12 or 24 h at 37 °C. In each case the cultures were adjusted to contain 2.5×10^7 cells/ml, 140 ml samples were centrifuged and the cell pellets resuspended in 5 ml of 0.01 M-potassium phosphate pH 5.5. These were used as whole cell suspensions, or cell-free extracts were prepared.

Table 3. *Glucanase assay of 12 h cultures of C. diffluens*

One ml of either cell suspension or extract was added to 1 ml 0.0066 M-*p*-nitrophenyl- β -D-glucoside and incubated at 30 °C.

	Units of enzyme*/ml	
	Samples from 37 °C	Samples from 27 °C
Intact cell	350	230
Culture filtrate	23	23
Cell extract	570	500

* The number of nmoles of product formed per hour in the assay.

Table 4. *Glucanase assay of 24 h cultures of C. diffluens*

For details, see Table 3.

	Units of enzyme*/ml	
	Samples from 37 °C	Samples from 27 °C
Intact cell	517	350
Culture filtrate	35	23
Cell extract	350	280

The results of the glucanase assays which were performed on these preparations (Tables 3 and 4) indicate that there is no significant difference in the level of glucanase in the samples derived at 27 and 37 °C. The results with the whole cells indicate that the 37 °C cells are more active than the 27 °C cells. However, the 37 °C cells are larger, and since an approximately equal number of cells were added per assay a somewhat higher activity should be expected with these cells (Dabbagh *et al.* 1974).

Although the foregoing results suggest that the level of glucanase in *C. diffluens* growing at 27 °C or exposed to 37 °C is the same, the possibility remained that the activity of this enzyme at 37 °C could be several times greater than its activity at 27 °C. Therefore the temperature coefficient for this enzyme was estimated by determining the rate of release of *p*-nitrophenol from the substrate at 27 and 37 °C. The rate of the reaction at 37 °C was twice that at 27 °C. It is concluded that the Q_{10} value for glucanase is not extraordinarily high but is consistent with the value found for most enzymes.

It is known in the case of many micro-organisms that the perturbation of wall biosynthesis is accompanied by the accumulation of wall precursors (Park & Strominger, 1957). These precursors are usually nucleotides. It was reasoned that if the temperature-induced effect in *C. diffluens* is because of hyperactivity of one or more of the enzymes responsible for wall biosynthesis, then wall precursors might accumulate in cultures exposed to the non-permissive temperature. Consequently, an extensive investigation of both the extracellular and intracellular nucleotides of *C. diffluens* at 27 and 37 °C was undertaken. The techniques employed were designed to detect uridine diphosphoglucose, a known precursor of wall biosynthesis in yeast. Fig. 1 shows the u.v. absorption spectra of a filtrate from a culture of *C. diffluens* grown at 27 °C in minimal medium plus 20 % sucrose, and a filtrate from the same culture that had been incubated at 37 °C for 48 h. The spectrum of the 37 °C filtrate shows a large peak at 262 nm and a smaller peak at 310 nm, but the 27 °C filtrate showed very little u.v.-absorbing material. These results indicate that at 37 °C *C. diffluens*

Fig. 1. Ultraviolet absorption spectra of filtrates obtained from cultures of *C. diffluens* grown at 27 and 37 °C on minimal medium containing 20 % sucrose. The blank contained fresh minimal medium; for other details see text.

Fig. 2. Ultraviolet absorption spectrum of fraction I from charcoal column.

excretes relatively large quantities of materials with the u.v. absorption characteristics at 262 nm of nucleotides. Also, since the 37 °C culture filtrate shows little 280 nm absorbing material, the 262 nm peak is not the result of cell lysis but of the excretion of low molecular weight compounds. In an attempt to purify the 262 nm absorbing material, the 37 °C culture filtrates were fractionated on a charcoal column. The column was eluted with 50 % ethanol adjusted with ammonium hydroxide to pH 9.0. The eluate was dried, dissolved with water to the original volume and adjusted to pH 7.0. Figure 2 shows the u.v. absorption spectrum of this fraction (fraction I). The material showed absorption peaks at 270 nm and 300 nm. Fraction I was found to contain pyruvate or other material which served as substrate for lactate dehydrogenase; absorption at 300 nm is consistent

Fig. 3. Ultraviolet absorption spectrum of fraction II from charcoal column.

Table 5. *Paper chromatography of fraction II from charcoal column*

Standard	<i>R_F</i> values in solvent systems	
	1	3
UDPG	0.16	0.71
AMP	0.45	0.31
UMP	0.23	0.67
Unknown spot		
1	0.16	0.72
2	0.45	0.31
3	0.23	0.67

with the presence of pyruvate. The same column was eluted with 50 % ethanol adjusted to pH 11 with ammonium hydroxide. This fraction (fraction II) was treated as above and the u.v. spectrum was determined. Fig. 3 shows that fraction II had an absorption peak at 258 nm. The spectrum of the original culture filtrate could be reconstructed by mixing fractions I and II; this indicates that all of the u.v.-absorbing material which was added to the column was recovered in these two fractions.

These spectral analyses were performed to determine whether uridine diphosphoglucose could be detected in the 37 °C culture filtrates. Although the spectrum of the crude filtrate exhibited an u.v. absorption consistent with that for UDP-glucose, the fractionated material did not. However, the presence of relatively small amounts of this wall biosynthetic intermediate may have been obscured by other nucleotides. Therefore, the crude filtrate and the two fractions obtained from the charcoal column were assayed for the presence of UDP-glucose. This assay took advantage of the high specificity of the enzyme UDP-glucose dehydrogenase, and using this enzyme it was demonstrated that the crude filtrate and fraction II contained UDP-glucose. The dehydrogenase assays were monitored until no further increase in NADH was observed. This level of NADH was then used as a measure of the concentration of UDP-glucose present in the samples. It was shown that the crude filtrate contained 3.2×10^{-3} μ mol UDP-glucose/ml, whereas fraction II contained 5.7×10^{-3} μ mol. The reason for the discrepancy between the levels of this nucleotide in the crude filtrate and fraction II is not known. The dehydrogenase reaction was also

Fig. 4. Ultraviolet absorption spectra of chromatographically purified material tentatively identified as uridine monophosphate. The sample was eluted from a chromatogram as described in the text; one portion was adjusted to pH 7 another to pH 2.

Fig. 5. Ultraviolet absorption spectra of chromatographically purified material tentatively identified as adenine monophosphate. The sample was eluted from a chromatogram as described in the text; one portion was adjusted to pH 7 and another to pH 2.

Fig. 6. Ultraviolet absorption spectra of the filtrate of a culture of (A) *C. diffluens* grown at 37 °C for 48 h in minimal medium supplemented with 20 % sucrose and (B), material released by boiling 27 °C-derived cells.

performed on concentrated crude filtrates obtained from 27 °C cultures of *C. diffluens*. These filtrates were completely inactive in the assay. Fraction II was analysed by paper chromatography. The results of this analysis are shown in Table 5. Four u.v.-absorbing spots were evident in each of the two solvent systems used. Several standards were also run in the same solvents. Tentative identifications (shown in the Table) were made on the basis of standard R_F values. One of the four spots on each chromatogram did not correspond to values obtained from standards or from reported values. The predominating spots corresponded to adenylic acid and uridylic acid. The remaining spot, which was present at much lower concentration, corresponded to UDP-glucose. The major spots were recoverable by elution with 50 % ethanol pH 11; the minor spot was not recoverable. Figs. 4 and 5 show the spectra of the eluted major spots at two pH values. These spectra with their characteristic pH dependence support the identification of the major nucleotide on UMP and AMP respectively.

The foregoing analysis illustrates that nucleotides, including UDP-glucose, were excreted by *C. diffluens* at 37 but not at 27 °C. The question arises as to whether the appearance of these nucleotides represents a cause or an effect with respect to the phenomenon under investigation. For example, the condition of the stabilized *C. diffluens* cells at 37 °C might have fostered a non-specific excretion of the contents of normal intracellular pools. To test this possibility, cells obtained from a 27 °C culture were briefly treated in a boiling-water bath, the cells removed by centrifugation and the spectrum of the supernatant fluid compared with that of the crude 37 °C culture filtrate. Fig. 6 shows that the two spectra are very similar, suggesting that the contents of the 37 °C filtrate may be a consequence of non-specific excretion. However, because the higher values from the 37 °C filtrate may have been caused by the presence of UDP-glucose, the material released from the 27 °C-grown cells was analysed for the presence of this compound. None was found. These

Fig. 7. Effect of source of inoculum on residual growth of *C. diffluens* at 35 °C. Cells were grown in minimal medium at 27 °C before inoculation of (○) minimal medium and (●) enriched medium at 35 °C.

results suggest that the nucleotides, including UDP-glucose, appear in the 37 °C filtrate as a consequence of nonspecific excretion. If this is so, the presence of UDP-glucose in the 37 °C filtrate should reflect the intracellular accumulation of this compound in cells at 37 °C. Therefore the differential intracellular accumulation of UDP-glucose was determined for cells growing at 27 °C and for stabilized cells at 37 °C. Cell extracts were prepared and the concentration of UDP-glucose was measured by the UDP-glucose dehydrogenase reaction. Cells from 70 ml of culture were harvested and suspended in 5 ml of 0.05 M-potassium phosphate pH 7.0. The cells were broken in a Mickle disintegrator. When portions of the extract were analysed with UDP-glucose dehydrogenase, UDP-glucose was not detected in the extract from the 27 °C cells. However, the extract from the 37 °C cells showed appreciable reactivity in the dehydrogenase reaction, the extract of the 37 °C cells containing $1.2 \times 10^{-2} \mu\text{mol}$ UDP-glucose/ml. These results indicate that UDP-glucose is accumulated at the non-permissive temperature.

The foregoing results indicate that, of the reactions considered in wall biosynthesis in *C. diffluens*, the glucan biosynthetic system rather than the degradative system may be more directly related to the temperature effect.

Because UDP-glucose accumulates at the non-permissive temperature, one would predict that glycosyl transferase, the enzyme which transfers glycosyl residues to the growing glucan chain, might be relatively inactive at the high temperature (37 °C). This question could be approached directly by determining the relative activity of the transferase at the two temperatures. However, these assays are difficult to perform owing to the unavailability of an acceptor molecule. Also, assays of crude preparations of the enzyme are not reproducible owing to the high level of β -1,3-exoglucanase which is present in the extracts.

An additional observation which shows that wall biosynthesis occurs and presumably that the glycosyl transferase is active at higher temperatures, resulted from an experiment

Fig. 8. Effect of source of inoculum on residual growth of *C. diffluens* at 35 °C. Cells were grown in enriched medium at 27 °C before inoculation of (○) minimal medium and (●) enriched medium at 35 °C.

designed to test the effect of previous growth at permissive temperatures on residual growth at non-permissive temperatures. The generation times of *C. diffluens* on minimal and enriched media are 4 h and 2 h respectively. Cells from cultures which were grown at 27 °C in minimal or enriched media were transferred to minimal and enriched media at 35 °C and the residual growth was followed. Fig. 7 shows that the same growth increment (2 generations) occurred in both media. Fig. 8 shows the results of a similar experiment, but with the inoculum prepared from enriched medium; the growth increment at 35 °C is equivalent to 3.5 to 4.0 generations. These results show that the residual growth upon shifting cultures from a permissive to a relatively non-permissive temperature was governed by the previous history of the inoculum rather than by the growth medium at 35 °C, and that wall biosynthesis and bud formation can occur at a relatively non-permissive growth temperature.

DISCUSSION

Evidence of abnormal activities of enzymes which have been implicated in bud initiation in other yeasts has not been obtained in *C. diffluens* at non-permissive growth temperatures. The indirect test for protein disulphide reductase used in this investigation could, for a variety of reasons, fail to reveal abnormal activity of this enzyme. However, although protein disulphide reductase has been found in the dimorphic fungus *Candida albicans*, it has not been conclusively demonstrated in a monomorphic budding yeast. In fact, Matile *et al.* (1971) have shown that the reductase is absent from *Saccharomyces cerevisiae*. The possibility remains that this enzyme is not present in *C. diffluens*.

A direct assay of β -1,3-glucanase, another enzyme which has been implicated in bud initiation and wall synthesis, showed that this enzyme is present in *C. diffluens*. Measurement of the activity of this enzyme in 27 and 37 °C-derived cells, as well as an estimation of its

temperature coefficient, suggests that the temperature-induced lysis could not be explained in terms of its hyper-activity. The glucanase assay used in this work measured β -1,3-exoglucanase, and it is possible that endoglucanase may be hyper-active at the higher temperature.

Non-specific excretion of low molecular weight compounds by *C. diffluens* exposed to 37 °C in minimal medium containing 20 % sucrose shows that these cells are unable to maintain their intracellular pools. It is not known whether this is a reflexion of increased permeability of the membrane *per se*, or whether it reflects an impairment of energy metabolism. The latter seems improbable, since general macromolecular synthesis continues for an extended time in sucrose-stabilized cells. We assume that other factors affecting membrane permeability are involved, which could also explain why the incorporation of endogenously supplied macromolecular precursors appears to cease before the delayed lysis of sucrose-stabilized cells (Dabbagh *et al.* 1974).

The presence of UDP-glucose in the filtrates of stabilized cultures reflects not only the relatively non-specific excretion of intracellular pools but also the intracellular accumulation of this compound specifically, at non-permissive temperatures; this contention is supported by the observation that UDP-glucose can be extracted from cells grown at 37 °C but not from cells grown at 27 °C. Accumulation of UDP-glucose can be explained in terms of either inaction of glycosyl transferase, the absence of appropriate acceptor molecules, or a derangement of the metabolic regulation of its synthesis.

The first possibility seems unlikely since it is known that cell enlargement occurs at 37 °C (Dabbagh *et al.* 1974) and also that bud formation occurs at a non-permissive temperature (35 °C); both of these processes require glycosyl transferase.

The correlation between the rate of growth of *Cryptococcus diffluens* and the extent of residual growth after a shift to 35 °C may be directly related to the temperature-induced aberration produced at 37 °C. The observation that cells growing rapidly at 27 °C exhibit more residual growth when placed at a non-permissive temperature than do slower-growing cells suggests that they are utilizing a constituent which was formed at 27 °C. The results indicate that the more rapidly growing cells possess more of this preformed substance or structure than do cells which are growing more slowly. The difference in the residual growth of the two types of inocula cannot be explained by differences in the pool size of a critical metabolic precursor. The dilution caused by the extent of residual growth (3 to 4 generations) of the faster-growing inoculum would require that the growth rate of the organism be relatively insensitive to the pool size of this hypothetical precursor. Utilization of a preformed structural component at the non-permissive temperature might be involved. For example it is possible that the number of bud initials on fast-growing cells of *C. diffluens* would be greater than on slow-growing cells. The fact that even rapidly growing *C. diffluens* cells have only a single bud does not argue against this possibility. The existence of multiple bud initials would be analogous to bacterial chromosome replication. It is known that under normal conditions chromosome replication is the rate-limiting process in bacterium division. It is also known that the chromosomes of more rapidly growing cells contain more growing points than do the chromosomes of cells growing less rapidly. Cooper & Helmstetter (1968) have presented a model of bacterial chromosome replication where the completion of a round of replication in one generation is the result of initiation in a previous generation. This allows more rapid chromosome replication. Bud formation in rapidly-growing *C. diffluens* could be analogous, with bud initials of varying maturity present on the mother cell, thus allowing more rapid budding by a newly born daughter cell. If the foregoing speculations are true then the bud site

produced at 37 °C must be of such a quality that it cannot serve as a primer for the UDP glucose-glycosyl transferase reaction, and the lack of acceptor would explain the accumulation of UDP-glucose at non-permissive temperatures.

At this point it is tempting to conclude that the pores which appear in stabilized cultures at non-permissive temperature are distended, abortive budding sites (Dabbagh *et al.* 1974). The budding process could then be considered as an extrusion of the protoplast through an incipient bud site with simultaneous wall biosynthesis. The lack of concomitant wall synthesis would result in lysis of the protoplast; this would explain why such rapid lysis occurs in non-osmotically stabilized cells and why protoplasmic growth precedes pore formation in stabilized cultures.

An alternative hypothesis is that the aberrant budding sites represent a temperature-induced derangement of the topological relationship of the cell membrane and the primitive bud pore. It is known that cell-wall-biosynthesizing enzymes, at least in bacteria, are membrane-associated (Ghuysen, Strominger & Tipper, 1968). Therefore, conformational changes in the membrane could lead to an uncoupling of the primer (cell wall) and donor (enzyme) reaction.

It is clear that further work must be done before the molecular mechanisms involved in the temperature-induced transitions in the saprophytic cryptococci can be described. However, the results of this and the accompanying paper (Dabbagh *et al.* 1974) show that the classical character of the saprophytic cryptococci, namely inability to grow at 37 °C, is the result of temperature-induced lysis which is probably caused by defective wall biosynthesis.

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