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Changes in the Rate of Chitin-plus-Chitosan Synthesis Accompany Morphogenesis of *Mucor racemosus*

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The in vivo differential rates of chitin-plus-chitosan biosynthesis in *Mucor racemosus* were determined under a variety of conditions, leading to yeast cell or mycelial morphology. Chitin-chitosan was determined as hot NaOH-insoluble radioactivity derived from *N*-acetyl-D-[1-^{3}H]glucosamine in the medium. Control experiments demonstrated that the labeled material possessed the properties of chitin-plus-chitosan. Our results indicate that *Mucor* yeasts have a relatively low differential rate of chitin-plus-chitosan synthesis and that mycelial cells have a threefold-elevated differential rate. Treatment of aerobic cells with exogenous N^6,O^2-dibutyryl cyclic adenosine 3',5'-monophosphate, an agent which induces yeast cell morphology, also results in a lowered rate of chitin-plus-chitosan synthesis. Control experiments eliminated the possibility that the observed rate changes were due to changes in endogenous pool size, uptake of exogenous *N*-acetyl-D-[1-^{3}H]glucosamine, or alterations in growth rate. Therefore, the changes are seemingly linked to morphogenesis. These results strengthen the idea that cyclic adenosine 3',5'-monophosphate plays an important role in dimorphism in *Mucor*. In addition, pulse-chase experiments suggest that considerable modification of newly synthesized chitin plus chitosan in both yeast cells and mycelia occurs in vivo.

Filamentous fungi grow by apical extension at the hyphal tip (6, 14). This property is believed to be the result of the fusion of vesicles containing wall synthesizing enzymes with the plasma membrane at the apex (3, 11, 12). Bartnicki-Garcia and co-workers (4, 7) have purified chitin synthetase-containing vesicles (chitosomes) from several diverse fungi. A few fungi are dimorphic, i.e., they are capable of growth as unicellular yeast cells or in a filamentous (mycelial) morphology. In dimorphic *Mucor* spp., yeast cell and mycelial walls are chemically similar (2). In the yeast form, wall polymers are laid down uniformly over the entire cell surface (6), leading to a spherical morphology. Presumably in yeast cells, chitosome fusion with the plasma membrane is not restricted to specific points as in the mycelial form.

An alternative mechanism for the localization of wall synthesis comes from the work of Cabib and associates. They have proposed (8, 9) that in *Saccharomyces*, chitin synthetase is uniformly distributed within the plasma membrane in an inactive zymogenic form. Localized activation of the zymogen by proteases carried to specific sites by vesicles is proposed to lead to chitin deposition at restricted areas (primary septa). Whatever the mechanism, it is clear that the spatial regulation of fungal wall growth is extremely precise.

Several lines of evidence have implicated cyclic adenosine 3',5'-monophosphate (cAMP) in the regulation of wall growth in fungi. In *Mucor* elevated intracellular levels of cAMP correlate with the yeast cell morphology. The addition of exogenous N^6,O^2-dibutyryl cAMP or cAMP to mycelial cells causes a growth-dependent conversion to yeasts (15, 18). In *Phycomyces*, changes in cAMP levels are associated with a phototropic response, the growth of sporangiophores toward light. It has been suggested that cAMP mediates the differential wall growth involved in the response. Exogenous cAMP results in restricted apical growth, increased hyphal branching, and thickening of the cell wall (24). Similar morphological consequences result from mutations in *Neurospora* which lead to decreased levels of cAMP. The addition of cAMP or cAMP analogs to these mutants at least partially reverses the morphological abnormalities (20, 22). Other fungi in which cAMP has been implicated in morphogenesis include *Histoplasma* (M. Sacco, B. Maresca, B. V. Kumar, G. Medoff, and G. S. Kobayashi, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, F24, p. 323), *Blastocladiella* (10), *Aspergillus* (27), and *Co-*

prinus (25).

Pall and Trevillyan have proposed that in the fungi cAMP is linked to fundamental properties of the cell membrane (17, 23). They have demonstrated that agents which depolarize the plasma membrane lead to an increase in intracellular cAMP levels. They suggest that the increased cAMP mediates, by unknown mechanisms, altered transport properties, changes in the rate of cell wall synthesis, and other cell surface properties.

In this paper, the in vivo rates of chitin-plus-chitosan biosynthesis in the dimorphic fungus *Mucor racemosus* are described. We demonstrate that the mycelial form of the fungus exhibits an elevated differential rate of synthesis compared with that of the yeast cell form. In addition, we show that exogenous cAMP, an agent which induces yeast cell morphology, results in the differential rate of synthesis characteristic of the yeast cell form. These observations strongly support the concept that cAMP is involved in the regulation of both the site(s) and rate of cell wall synthesis. In addition, we show that considerable modification of existing chitin plus chitosan takes place during growth.

MATERIALS AND METHODS

Organism. *M. racemosus* ATCC 1216B was used throughout the experiments.

Media and culture conditions. All growth experiments were performed in a complex medium containing: 0.3% yeast extract, 1% peptone, and 2% glucose (YPG). The pH of the medium was adjusted to pH 4.5 with H_2SO_4. Unless stated otherwise, all media were supplemented with 300 μg of *N*-acetyl-D-glucosamine (GlcNAc) per ml. Anaerobic (yeast) growth in a CO_2-containing atmosphere was accomplished by sparging CO_2 through the medium at a rate of 1 ml of CO_2/ml of culture per min. Aerobic (mycelial) growth entailed sparging sterile air through the culture at 10 ml of air/ml of culture per min. Unless stated otherwise, starter cultures were inoculated with sporangiospores at a density of 2×10^5 spores per ml. Liquid cultures were incubated at 28°C with rotary shaking.

In experiments entailing CO_2-to-air shifts, yeast cell cultures were started from spores in YPG medium. After a 24-h growth period, the cells were diluted 1:10 into fresh medium. After 12 h of growth in CO_2, the culture was again diluted 10-fold into YPG medium with 0.5 μCi of [^{3}H]GlcNAc per ml. An additional 12 h of growth ensued, and the culture was diluted 1:10 into fresh YPG medium with 0.5 μCi of [^{3}H]GlcNAc per ml. After 4 h, the culture was divided into three portions: one remained in CO_2, one was shifted to aerobic conditions, and one was shifted to aerobic conditions plus 3 mM dibutyryl cAMP (dbcAMP). At 1-h intervals, triplicate 0.1-ml portions of the culture were removed to assay for chitin-plus-chitosan synthesis, and duplicate 0.5-ml portions were removed for protein determination.

Determination of chitin-plus-chitosan synthesis. Samples (0.1 ml each) of culture were suspended in 1 ml of 0.5 N NaOH, heated for 5 min in a boiling water bath, and cooled on ice. The mixture was filtered on 2.4-cm glass fiber filters (Whatmann, GFC) and washed three times with 3 ml of distilled water and then once with 10 ml of 95% ethanol. The filters were dried, placed in scintillation vials, and counted in a toluene-based scintillation fluid (4 g/liter; Omnifluor, New England Nuclear Corp.).

Protein determination. Culture samples were centrifuged and washed twice with 5 ml of distilled water. The cell pellet was suspended in 0.5 ml of 0.5 N NaOH and heated in a boiling water bath for 5 min. The mixture was centrifuged, and the supernatant was assayed for protein by the Lowry method (16).

Enzymatic digestion of chitin plus chitosan. YPG medium (10 ml) with 1 μCi of [^{3}H]GlcNAc per ml was inoculated with 10^6 spores per ml. The cells were grown for 10 h aerobically as mycelia. They were harvested, washed twice with distilled water, suspended in 3 ml of 0.5 N NaOH, and heated at 100°C for 5 min. After heating, the mixture was homogenized by sonication and divided into three aliquots in conical centrifuge tubes. Each aliquot was centrifuged and washed with 0.5 N NaOH and distilled water. The pellets were suspended in 1.50 ml of sodium phosphate buffer, pH 6.8. Crude chitinase (Calbiochem) and purified *Myxobacter* AL-1 chitosanase were added alone or in combination at final concentrations of 3 mg/ml and 300 U/ml, respectively. The tubes were incubated at 30°C with periodic agitation. At intervals, each sample was centrifuged at 12,000 × *g* and the radioactivity in 0.1 ml of the supernatant was determined. After samples had been removed, each tube was agitated by blending in a Vortex mixer to resuspend the precipitate.

Specific activity of [^{3}H]GlcNAc in chitin plus chitosan. The specific radioactivity of [^{3}H]-GlcNAc in chitin plus chitosan was determined by total acid hydrolysis of alkali-insoluble material derived from CO_2-grown yeast and aerobic cells during a CO_2-to-air shift. The alkali-insoluble material was prepared as for the digestion with chitinase and chitosanase but was washed finally with distilled water and suspended in 0.5 ml of 6 N HCl. The samples were hydrolyzed in evacuated, sealed vials at 110°C for 20 h. HCl was removed by rotary evaporation, and the glucosamine in the residue was determined on a Glenco MM-50 amino acid analyzer. Effluent samples from the analyzer were collected in scintillation vials and counted after adding 10 ml of Scinti Verse (Fischer Scientific Co.).

Gel filtration chromatography. Pulse-chase products were analyzed, before and after enzymatic digestion, on a column (1.6 by 90 cm) of Bio-Gel P-2 (200 to 400 mesh). The column was equilibrated in water at a flow rate of 30 ml/h. Two-milliliter fractions were collected, and 0.2-ml aliquots were dried on glass fiber filters and counted.

Chitosanase purification. *Myxobacter* AL-1 chitosanase was purified to homogeneity by the method of Hedges and Wolfe (13).

RESULTS

Nature of the alkali-insoluble material. The material which is labeled by [^{3}H]GlcNAc

and is insoluble after hot NaOH treatment has the properties expected of chitin and chitosan. Figure 1 shows the results of digestion of the precipitate with crude chitinase, homogeneous *Myxobacter* AL-1 chitosanase, and a combination of the enzymes. Essentially 100% of the radioactivity can be solubilized by chitosanase alone or by a combination of enzymes. Chitinase alone liberates only about 40% of the total counts. Total acid hydrolysis in 6 N HCl of the alkali-insoluble material, followed by amino acid analysis, indicated that 100% of the radioactivity was present in glucosamine. Small amounts of unlabeled amino acids were detected in the analyses. We conclude from these experiments that the hot NaOH assay measures chitin plus chitosan.

Balanced growth of yeast cell cultures in CO_2. Before CO_2-to-air shifts were performed, the culture was brought to balanced growth with respect to protein synthesis and [^{3}H]GlcNAc incorporation (Fig. 2). To achieve this effect, CO_2-grown yeast cells had to be precultured in YPG medium supplemented with a high concentration of GlcNAc (300 μg/ml) by the regimen presented above. In experiments in which incorporation was measured without repeated subculturing, the rate of [^{3}H]GlcNAc incorporation was greater than that of protein accumulation. Since we used a wild-type strain (i.e., nonauxotrophic for GlcNAc) for these experiments, the long period of logarithmic growth and high concentration of GlcNAc may be necessary to maximally repress the endogenous pathway of GlcNAc synthesis.

Rate of chitin-plus-chitosan synthesis during a CO_2-to-air shift. Figure 3 shows the results of an experiment in which a culture of

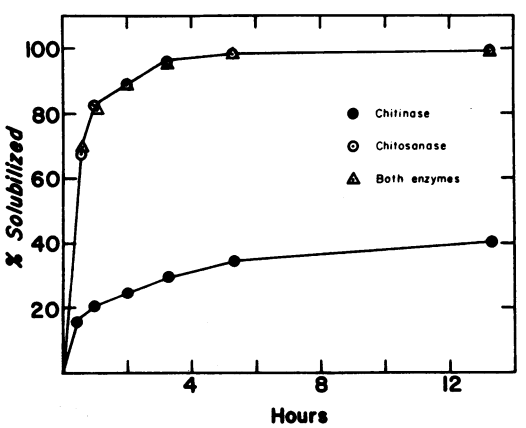

FIG. 1. *Digestion of [^{3}H]GlcNAc-labeled, alkali-insoluble material with crude chitinase (3 mg/ml), isolated Myxobacter AL-1 chitosanase (300 U/ml), and a combination of the enzymes.*

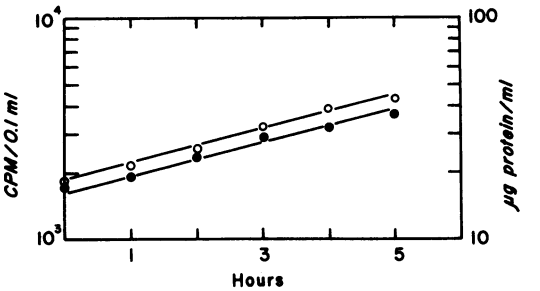

FIG. 2. *Balanced chitin-plus-chitosan synthesis and protein synthesis in CO_2-grown yeast cells. YPG medium (20 ml) was inoculated with 2×10^5 spores per ml which were grown for 24 h at 28°C in a CO_2 atmosphere. The culture was diluted 1:10 in the same medium and was again incubated under CO_2 for 12 h. The culture was again diluted 1:10 in the same medium but 0.5 μCi of [^{3}H]GlcNAc per ml was added. Finally, after 12 h of incubation, another 1:10 dilution into YPG medium with 0.5 μCi of [^{3}H]GlcNAc per ml was performed, and the culture was incubated at 28°C under CO_2. Protein content (●) and [^{3}H]GlcNAc content in chitin plus chitosan (○) were monitored.*

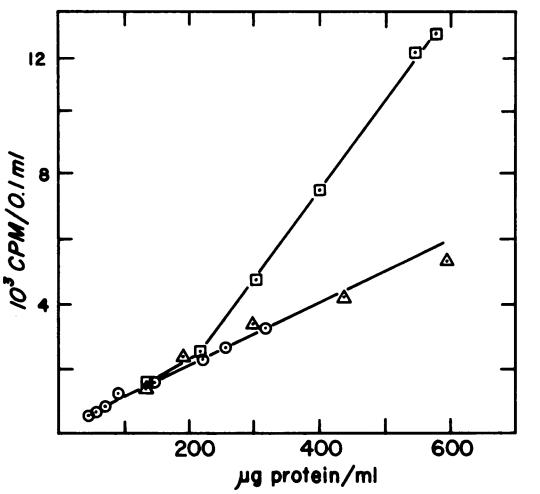

FIG. 3. *Differential rate of chitin-plus-chitosan synthesis during a CO_2-to-air shift. A yeast cell culture in balanced growth was labeled in YPG medium with 0.5 μCi of [^{3}H]GlcNAc per ml. At the point represented by the arrow, the culture was divided: one portion remained in CO_2 (⊙); one portion was made aerobic (⊡); and the third portion was made aerobic with dbcAMP being added to a final concentration of 3 mM (△). Protein and the chitin-plus-chitosan content of the cells were monitored.*

CO_2-grown yeast cells in balanced log growth was divided, and individual portions were: (i) made aerobic, (ii) made aerobic in the presence of 3 mM dbcAMP, and (iii) allowed to remain in the CO_2 atmosphere. At about 3 h postshift, the aerobic cells began to develop hyphae; the CO_2 cells and aerobic cells in the presence of

dbcAMP remained yeastlike. Both of the aerobic cultures showed an elevated growth rate (ca. 1.5-h doubling time versus ca. 4-h doubling time for CO_2 cells). It is evident from the data that the differential rate of chitin-plus-chitosan synthesis increases about threefold in aerobic culture (mycelia), whereas those of the CO_2 (yeast cell) and air-plus-dbcAMP (yeast cell) cultures remain at the preshift differential rate. The increase in the rate in the aerobic culture occurs between 2 and 3 h postshift, a point in time which coincides or slightly precedes the emergence of hyphal tubes.

Rate of chitin-plus-chitosan synthesis during an air-to-CO_2 shift. Figure 4 shows the differential rates of chitin-plus-chitosan synthesis accompanying an air-to-CO_2 shift. YPG medium with 0.5 μCi of [^{3}H]GlcNAc per ml was inoculated with 2×10^5 spores per ml and incubated aerobically. After 6 h, the culture was divided (germ tubes had just appeared). Two portions remained aerobic; one of these received dbcAMP to a final concentration of 3 mM. The third portion was shifted to a CO_2 atmosphere.

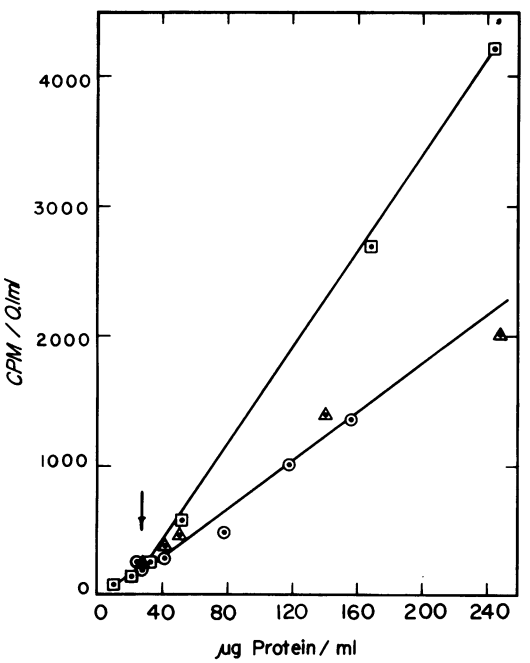

FIG. 4. *Differential rate of chitin-plus-chitosan synthesis during an air-to-CO_2 shift. An aerobic culture was inoculated with 2×10^5 spores per ml in YPG medium supplemented with 0.5 μCi of [^{3}H]-GlcNAc per ml at 28°C. After 6 h of growth, the culture was divided: one portion remained aerobic (□); one portion remained aerobic, and dbcAMP was added to a final concentration of 3 mM (△); and the third portion was shifted to a CO_2 atmosphere (⊙). Protein and chitin-plus-chitosan content of the cells were monitored.*

Chitin-plus-chitosan synthesis and protein were monitored. At the end of the experiment (4 h postshift), the CO_2 culture and the aerobic culture plus dbcAMP were predominantly yeastlike; the aerobic culture was filamentous. Once again, the differential rate of chitin-plus-chitosan synthesis was about threefold lower in the two yeastlike cultures.

Specific activity of chitin plus chitosan. During a CO_2-to-air ± dbcAMP shift experiment, cell samples were removed just before the shift and at 4 h postshift to determine the specific radioactivity of [^{3}H]GlcNAc in chitin plus chitosan. If the relative specific activity of the preshift CO_2 cells was set to 1.00, the relative specific activity of the aerobic (dbcAMP) yeast cell culture was 1.20 and that of the aerobic mycelia culture was 1.30. This suggests that slight changes in uptake or endogenous pool size occur during the shift, but the magnitude of the changes is insufficient to account for the observed changes in the differential rate of synthesis.

Pulse-chase experiments. Figure 5 shows the chase kinetics from logarithmically growing CO_2-grown yeast cells and aerobic mycelia pulsed with [^{3}H]GlcNAc for 30 min and 40 min, respectively. Somewhat surprisingly, radioactivity is shown to be chased from chitin plus chitosan under both aerobic and anaerobic condi-

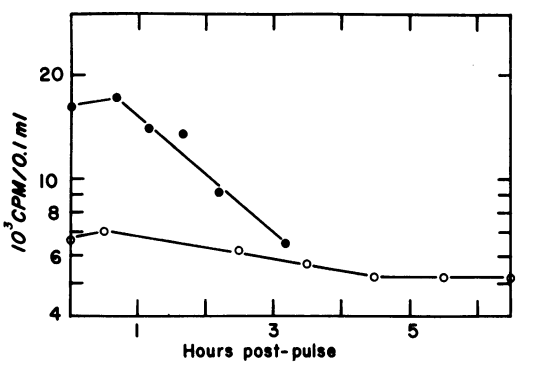

FIG. 5. *Chase kinetics in CO_2-grown yeast cells and aerobic mycelia. A CO_2-grown yeast cell culture in balanced log growth in YPG medium plus 10 μg of GlcNAc per ml was pulsed with 5 μCi of [^{3}H]GlcNAc per ml for 30 min. Cells were centrifuged, washed, and resuspended in YPG medium plus 300 μg of GlcNAc per ml. An aerobic culture inoculated with 2 $\times 10^5$ spores per ml in YPG medium plus 50 μg of GlcNAc per ml was pulsed 8 h after inoculation with 10 μCi of [^{3}H]GlcNAc for 40 min. The cells were centrifuged, washed, and resuspended in YPG medium plus 300 μg of GlcNAc per ml. Chitin-plus-chitosan contents of the yeast cells (○) and mycelia (●) were monitored during the chase by the hot NaOH assay.*

tions. In aerobic mycelial cells, the process is more rapid and extensive, with about 40% of the label remaining in alkali-insoluble material after 4 h (2.7 doublings). The process did not slow by the end of the chase period. In CO_2-grown yeast cells, radioactivity in chitin plus chitosan declined to about 70% of the original after 4.5 h and stabilized. In later experiments (data not shown), it was found that the rate of decay of radioactivity was independent of the concentration of GlcNAc in the chase medium.

Nature of the chase products. An aerobic culture (10^6 spores per ml of inoculum) growing on YPG medium supplemented with 50 μg of GlcNAc per ml was pulse-labeled at 8 h after inoculation for 40 min with 5 μCi of [^{3}H]GlcNAc per ml. After the labeling period, the cells were washed with and resuspended in fresh YPG medium supplemented with 300 μg of GlcNAc per ml. After 2 h in the chase medium, the cells were harvested by centrifugation, washed with distilled water and broken by sonication. Cell walls were removed by centrifugation at 12,000 × *g* for 10 min, and the supernatant was collected for analysis of soluble chase products. The chase products were prepared by this method to avoid possible alkaline degradation. Table 1 shows the fate of the radioactivity during the experiment. Essentially 100% of the counts incorporated into chitin plus chitosan during the pulse were recovered. The results strongly suggest that all of the radioactivity obtained in the soluble fraction after the chase period was derived from chitin plus chitosan. In addition, they imply that [^{3}H]GlcNAc is not incorporated to any significant extent in other stable macromolecules (relative to chitin plus chitosan) during the pulse period. The data also support the validity of this method of preparation as compared with the hot NaOH assay.

A portion of the soluble chase products was chromatographed on Bio-Gel P-2 before and after digestion with chitinase and chitosanase. The results of the chromatography are shown in Fig. 6. Undigested material eluted in the void volume. In separate experiments, we have shown that the chase products also elute in the void volume of Bio-Gel P-4 and P-30. Therefore, the material is macromolecular. Exhaustive chitinase and chitosanase digestion did not result in total degradation of the material. In the experiment shown in Fig. 6, about 75% of the material was converted to products which could enter the gel. The major peak included in the gel corresponded to the approximate elution position of a disaccharide.

TABLE 1. *Analysis of chase products*

Step of experiment	cpm/ml of culture	% Original incorporation
1. Hot NaOH insoluble after 40-min pulse	1,066,200	100[a]
2. Hot NaOH insoluble after 2-h chase	844,110	79.2
3. Medium after 2-h chase	2,500	0.2
4. Supernatant after sonication	214,000	20.1
5. Pellet after sonication	842,200	79.0
6. Sum of 3, 4, and 5	1,058,700	99.3

[a] Set to 100% incorporation.

DISCUSSION

Despite considerable interest in the biosynthesis of fungal cell walls in the past 15 years, relatively little information concerning the in vivo rates of wall polymer synthesis is available. This is especially surprising for chitin, which is believed to be the most important skeletal polysaccharide in the primary wall of many diverse fungal groups (2). The biosynthesis of chitin and the enzymes which modify and degrade it has been an area of active research; however, by far

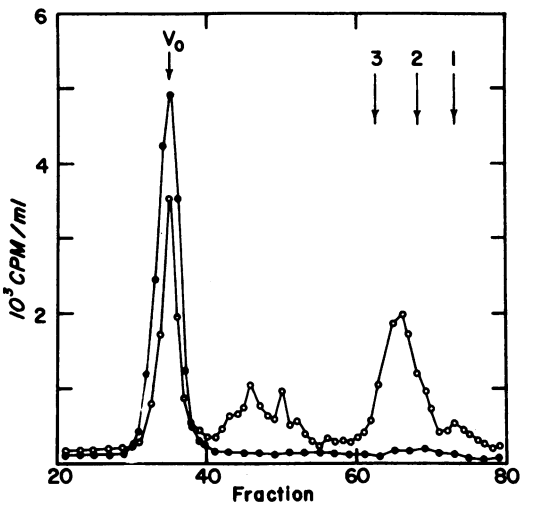

FIG. 6. *Analysis of chase products on a Bio-Gel P-2 column (1.6 by 90 cm). The column was equilibrated at 30 ml/h with water; 2-ml fractions were collected. Symbols: (●) profile before chitinase-plus-chitosanase digestion; (○) profile after chitinase-plus-chitosanase digestion. Chase products were digested for 18 h at 30°C in 1 ml of 0.02 M sodium phosphate buffer (pH 6.8) with additions of crude chitinase (5 mg) and purified chitosanase (100 U) at 0 h and again at 8 h. Numbered arrows refer to the elution positions of mono-, di-, and trisaccharide standards. V_0 is the elution position of a void volume marker. Approximately 20,000 cpm of undigested material was applied to the column; 40,000 cpm of chitinase-plus-chitosanase-digested material was applied in the parallel run.*

most of the experimental data have been obtained from in vitro investigations. The in vivo experiments which have been performed have usually involved autoradiography of cells after a pulse-labeling period with wall precursors. These experiments, although quantitative, had as their objective a definition of the localization of the site(s) of wall synthesis. In addition, this technique (autoradiography) is not useful for routinely monitoring wall synthesis during fungal growth and morphogenesis.

In this paper we have demonstrated that in vivo rates of chitin and chitosan (the deacetylated form of chitin) can be routinely monitored by simple methods in *M. racemosus* cells labeled with [^{3}H]GlcNAc. The assay is dependent on the insolubility of chitin and chitosan in hot alkali and the solubility of most other macromolecules under these conditions. The alkali-insoluble product(s) has the properties of chitin and chitosan in that it is degraded by chitinase and chitosanase. In addition, acid hydrolysis of the alkali-insoluble products yields label only in glucosamine (GlcNAc is deacetylated during acid hydrolysis). At least in *M. racemosus*, a glucosamine or GlcNAc auxotroph is not a necessity for these experiments as long as the necessary controls are performed and the labeling is performed under the proper growth conditions. The alkali-insoluble radioactive polymer(s) produced by the hot NaOH assay can be solubilized totally by purified chitosanase alone. This finding should be interpreted cautiously since chitin could be partially deacetylated by the alkaline isolation conditions, thereby rendering it susceptible to attack. Alternatively, chitin and chitosan may not be distinct polymers. It is possible that all chitin is randomly deacetylated by chitin deacetylase (1), hence leading to a heteropolymer which can be solubilized by chitosanase.

All of our experiments have been performed in cells in logarithmic growth. In addition, in experiments involving CO_2-to-air shifts, cells were first brought to balanced growth. Gooday and Trinci (12) have emphasized the importance of balanced growth and exponential cultures for studies of wall synthesis and composition. Unfortunately, many investigators have ignored these points, making detailed comparisons of experiments from different laboratories difficult, if not impossible.

Chitin and chitosan deposition in mycelial cells of *Mucor* spp. occurs predominantly, as in other filamentous fungi, at the hyphal apex (6). Unlike most other fungi, however, an alternative mode of wall growth exists in dimorphic *Mucor* spp. In yeast cells, chitin synthesis is unpolarized (6), giving rise to almost perfectly spherical cells which divide by budding. The nature of the polarizing mechanism in fungi is unknown. Among the agents which cause a hyphal-to-yeast cell morphogenesis in *Mucor* spp., cAMP (18) and dbcAMP (15) are the most fascinating. It follows that since these nucleotides evoke yeast cell morphogenesis, they affect at least the sites of wall synthesis. Whether this is a direct or indirect effect on the polarizing mechanism remains to be seen. Our results indicate that in addition to influencing the sites of chitin-chitosan synthesis, dbcAMP affects the rate of chitin-chitosan synthesis. Yeast cells have a characteristically lower rate of chitin-chitosan synthesis than do mycelial cells. In aerobic cells treated with dbcAMP, the characteristic yeast cell rate of synthesis is maintained. That the lowered rate of chitin-plus-chitosan synthesis is unique to yeast cell morphology and not to slower growth rate in CO_2 is argued by the fact that aerobic mycelia grow at the same rate as do aerobic yeast cells (treated with dbcAMP). Thus, aerobic cells treated with dbcAMP mimic the physiological (rate of chitin-plus-chitosan synthesis) and morphological properties of anaerobic yeast cells generated in the absence of dbcAMP. In addition, another physiological property of yeast cells, the inability to grow on disaccharides, is evoked by cAMP (5). In total, these experimental findings strengthen the concept that cyclic nucleotides play an important role in dimorphism.

The level at which cAMP affects chitin-plus-chitosan synthesis is of course unknown. Chitin synthase in most fungi examined is believed to be synthesized as a zymogen (4, 8, 12) which must be proteolytically activated. In many fungi, the zymogen is present in cytoplasmic membranous microvesicles (chitosomes) (4, 7), which presumably fuse with the plasma membrane at the hyphal apex. Our results do not eliminate any of the potential levels for regulation of chitin synthase. Therefore, an increase in enzyme protein is possible (transcriptional or translational regulation). Alternatively, a posttranslational level, such as phosphorylation, proteolytic activation, vesicle fusion, or allosteric regulation, could be the level of cAMP action. It is possible that cAMP does not directly affect the activity of chitin synthase. If cAMP were only involved in the regulation of the sites of wall synthesis (i.e., in turning off the polarizing mechanism), then decreased chitin synthase activity might result simply from the reduced surface/volume ratio in the spherical yeast cell.

The degradation of wall polymers in fungi has long been believed to be a necessary correlate for wall growth and subapical branch formation (3, 11, 21). However, in many instances wall

turnover is difficult to demonstrate. Polachek and Rosenberger (19) could not demonstrate turnover in pulse-chase experiments in *Aspergillus nidulans*. Our observations that radioactivity can be chased from chitin plus chitosan should be interpreted cautiously. It should be emphasized that we have not demonstrated polymer turnover, in that low-molecular-weight chase products were not detected. Rather, we find that polymers with modified properties are derived from chitin plus chitosan. These properties include: (i) the chase products are not filterable on glass fiber filters; (ii) they do not sediment at 12,000 × *g* in 10 min; and (iii) they cannot be totally degraded by exhaustive digestion by chitinase and chitosanase. These properties suggest that rather than extensive depolymerization, the products are the result of modification. Wessels and Sietsma (26) have suggested that in *Schizophyllum communae*, chitin is covalently linked to glucan. Additionally, the free amino group of glucosamine in chitosan could form covalent linkages to polypeptides or proteins. Small amounts of amino acid can usually be detected in chitin plus chitosan even after drastic extraction procedures. These kinds of linkages might play a role in the interactions of wall polymers and be important to the plasticizing of the wall necessary for growth.

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