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Inhibitory Effect of Cerulenin and Sodium Butyrate on Germination of *Candida albicans*

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Candida albicans germination in liquid medium was inhibition by the antilipogenic agent cerulenin and the fatty acid sodium butyrate. Although these inhibitors prevented germ tube emergence at concentrations of 1 μg/ml and 20 mM, respectively, neither significantly affected cell viability as judged by trypan blue staining or the rate of protein biosynthesis throughout the time course of the experiments. Cerulenin treatment resulted in inhibition of lipid biosynthesis, but lipid biosynthetic capabilities remained unaltered in sodium butyrate-supplemented cultures. Because each inhibitor blocks germination by different mechanisms, their utility in distinguishing events directly correlated to germination was examined. In this context, chitin synthase activity was inhibited by both compounds, confirming the importance of chitin biosynthesis in *C. albicans* germination.

Candida albicans is a dimorphic fungus capable of either yeast or mycelial growth, depending on environmental conditions. Although generally an unremarkable member of the human microbial flora, *C. albicans*, as an opportunistic pathogen, can promote infections ranging from superficial irritations to systemic disease (17). In this regard, investigations of *C. albicans* have focused on understanding dimorphism and its relationship to the invasiveness of the organism. Although the phenomenon of dimorphism is well known, the key biochemical and molecular events which regulate the transition of the yeast form to the hyphal form have yet to be identified.

One approach to examining these issues is to dissect the steps leading to germination by employing agents that inhibit the process. In the present study we investigated the effect of cerulenin and sodium butyrate on the germination of *C. albicans*. Cerulenin is an antibiotic that inhibits fatty acid biosynthesis in a number of bacteria, yeasts, and fungi (18). This property has been exploited to examine phospholipid and sterol functions in several systems. For example, cerulenin has been utilized to study the relationship between membrane biogenesis and spore germination in *Rhizopus stolonifer* (16) and *Botryodiplodia theobromae* (2). Furthermore, cerulenin has been used to uncouple phospholipid biosynthesis from other processes in the study of differentiation of *Dictyostelium discoideum* (4), *Caulobacter crescentus* (7), and *Mucor racemosus* (11). On the other hand, sodium butyrate has been used primarily in the study of higher eucaryotic cells and has been shown to reversibly influence a number of diverse processes, including cellular differentiation, cell growth, enzyme induction, and cellular morphology (12). The mechanism(s) by which sodium butyrate mediates these alterations has not been defined rigorously; however, the compound does not significantly affect RNA or protein synthesis in these systems.

In this communication, we report the utility of both cerulenin and sodium butyrate in the study of *C. albicans* germination. In particular, we demonstrate that use of both agents in combinations may provide a way to examine the importance of membrane biogenesis and function to morphogenesis.

MATERIALS AND METHODS

C. albicans 4918 was utilized in all experiments to prevent the complication of experiments by the appearance of pseudohyphae (14). Cells were maintained on GYE (2% glucose, 1% yeast extract, 2% agar) slants. Cultures were prepared by inoculating 50 ml of Phytone peptone broth (BBL Microbiology Systems) containing 1 mg of glucose per ml and incubating for 18 h at 28°C. Subsequently, cultures were washed three times with phosphate-buffered saline (0.12 M sodium chloride, 0.037 M sodium phosphate [pH 7.2]) (PBS) and standardized to a final concentration of 5×10^7 cells per ml. Germination was induced by shifting cultures to 37°C. For all experiments, quantities of standardized culture were centrifuged and resuspended in appropriate amounts of Phytone peptone broth. The extent of germination was monitored by

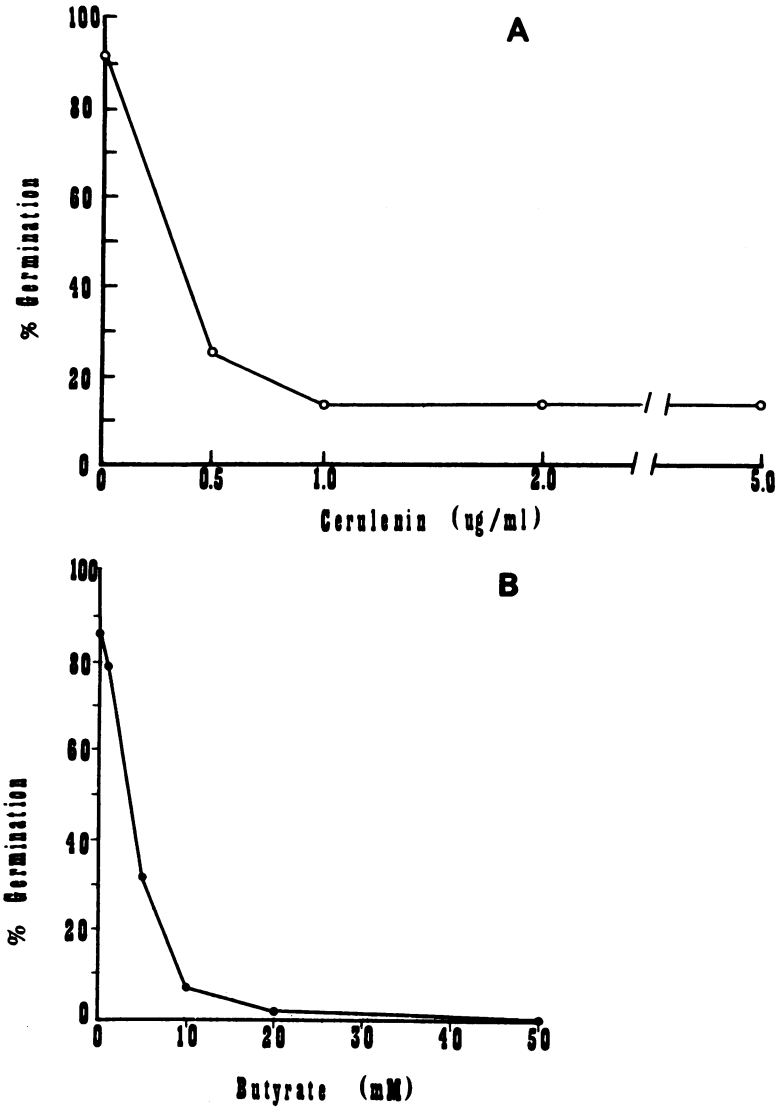

FIG. 1. Effect of cerulenin and sodium butyrate on *C. albicans* germination. Cerulenin (A) or sodium butyrate (B) was added to *C. albicans* cultures 30 min before the beginning of incubation of cells at 37°C, and the extent of germination was determined by microscopic observation after 3.5 h.

light microscopy. In experiments in which either cerulenin or sodium butyrate was utilized, the particular agent was added to yeast cultures maintained at 28°C 30 min before beginning incubation at 37°C.

Protein biosynthesis. Protein biosynthesis was quantitated by determining the amount of L-[4,5-^{3}H]leucine incorporated into trichloroacetic acid (TCA)-insoluble material. Samples of 1.0 ml were taken from 20 ml of cultures containing 2.5 μCi of L-[4,5-^{3}H]leucine at 20-min intervals and placed into 1 ml of 10% TCA. Subsequently, the samples were heated to 90°C for 20 min and then chilled for 30 min at 0°C. Precipitates were collected by filtration; filters were washed with 3 volumes of 10% TCA and 2 volumes of 95% ethanol, dried, and counted.

Lipid biosynthesis. Lipid synthesis was measured by assaying the incorporation of [^{3}H]acetate into methanol-chloroform-extractable material. [^{3}H]acetate at 1 μCi/ml was added to cultures at the onset of incubation at 37°C. Samples of 5.0 ml were removed at 15-min intervals into 5.0 ml of 10% TCA and incubated overnight at room temperature. TCA-precipitable material was collected by filtration and washed into test tubes with 2.0 ml of anhydrous methanol. After the addition of 0.5 g of glass beads, the mixture was vigorously agitated on a vortex mixer for 4 min.The material was next heated to 55°C for 15 min and allowed to cool to room temperature, after which 4 ml of chloroform was added to each sample. After overnight incubation at room temperature, the methanol-

chloroform-extractable material was filtered through glass fiber filters and extracted with 3 volumes of 2 M KCl–1 volume of distilled water. Samples of the filtrate were then removed to scintillation vials, dried, and counted in Aquasol.

Chitin biosynthesis. Chitin synthesis was assayed by determining the incorporation of [^{3}H]*N*-acetylglucosamine into acid-alkali-insoluble material (3). At appropriate times after the shift up to 37°C, cultures were pulsed for 20 min with 10 μCi of [^{3}H]*N*-acetylglucosamine per ml. Incorporation was stopped by the addition of 2 ml of 15% TCA to each 4-ml culture. Paired samples were taken from each culture (3 ml each); one of each pair was placed immediately at −70°C, and the other was filtered through a nitrocellulose filter (0.2-μm pore size; Nucleopore Corp.). Filters were then stored at −70°C. Cells from the latter samples were resuspended in PBS buffer and disrupted with glass beads, and total protein in each sample was then determined by the method of Lowry (13). The nonfiltered samples were thawed the next day, washed twice with PBS, resuspended in 2 ml of 1 N HCl, and boiled for 90 min. Samples were washed again, resuspended in 2 ml of 1 N KOH, and boiled for an additional 90 min. The cell pellets were finally washed and resuspended in 2 ml of PBS. Subsequently, the insoluble material was collected by filtration onto glass fiber filters and washed with 95% ethanol. After drying, filters were placed in 5 ml of Aquasol and counted. Data were normalized and expressed as cpm per microgram of protein.

RESULTS

Inhibition of germination by cerulenin and sodium butyrate. Preliminary experiments indicated that cerulenin and sodium butyrate could effectively prevent *C. albicans* morphogenesis. Cerulenin effectively inhibited germination at concentrations of as low as 1 μg/ml (Fig. 1A). An increased number of yeast cells germinated at low cerulenin concentrations, whereas increasing cerulenin concentrations to higher values did not further reduce the observed number of cells that began germination. Thus, all subsequent experiments were performed with cerulenin at 1 μg/ml. In the case of sodium butyrate, concentrations of 50 mM or greater completely abolished germination. When used at 20 mM, sodium butyrate allowed only 1 to 3% germination (Fig. 1B), and this concentration was used in all other experiments.

The morphology of cells treated with either of the two agents is shown in Fig. 2. In comparison with an untreated control (Fig. 2a), germ tube emergence was sharply inhibited when cells were treated with either cerulenin (Fig. 2b) or sodium butyrate (Fig. 2c). In addition, cells treated with sodium butyrate were somewhat larger than those treated with cerulenin and remained as chains resembling pseudohyphae.

Emergence of germ tubes ensued at ca. 90 min after a shift up in temperature (28 to 37°C) in control cultures and reached a maximum by 120 to 150 min. The results in Fig. 3 show the ability of cerulenin or sodium butyrate to inhibit germination when added at times after the temperature shift. Sodium butyrate prevented morphogenesis when added at times up to 30 min after

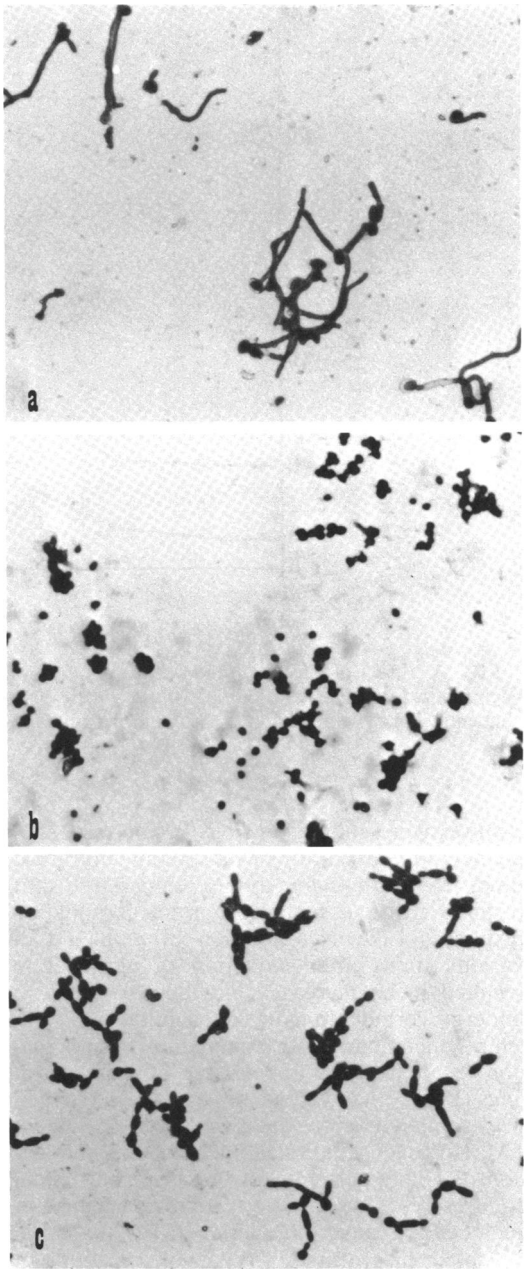

FIG. 2. Photomicrographs of *C. albicans* cells after 3.5 h of incubation at 37°C. (a) Untreated control; (b) cultures supplemented with 1 μg of cerulenin per ml 30 min before incubation; (c) cultures supplemented with 20 mM butyrate 30 min before incubation. Magnification, ×400.

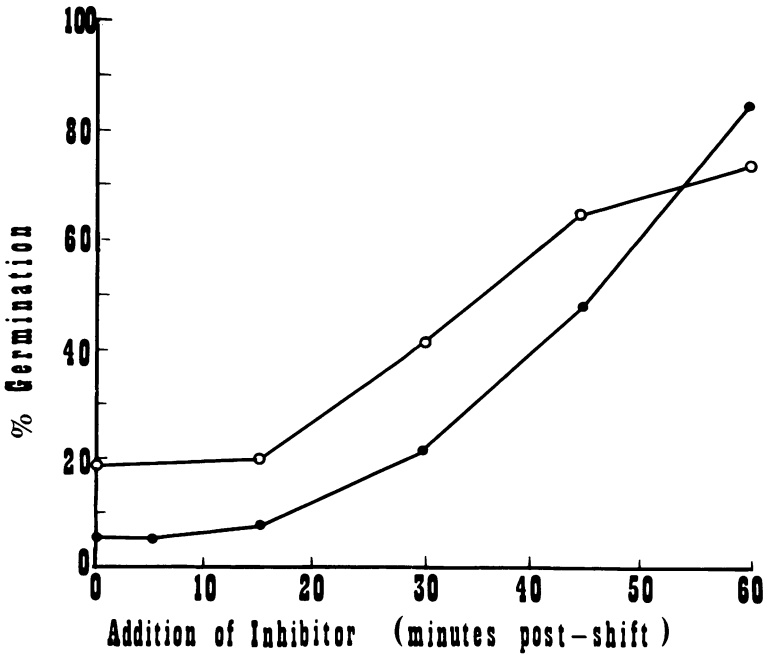

FIG. 3. Effect of cerulenin and sodium butyrate on *C. albicans* germination when added at times after the beginning of incubation at 37°C. Cultures were grown as described in the text and supplemented to a final concentration of either 1 μg of cerulenin per ml (○) or 20 mM sodium butyrate (●).

cultures were shifted to 37°C. In contrast, supplementation of cultures with sodium butyrate at times later than 45 min after a temperature shift resulted in an essentially normal germination response. Likewise, addition of cerulenin at various times after induction of germination resulted in an increased likelihood of cells to undergo germination. In fact, addition of cerulenin concomitant with stimulation of germination resulted in the appearance of more germlings (Fig. 3) than when cells were preincubated with cerulenin before the temperature shift (Fig. 1A). However, continued microscopic examination of young germlings from cerulenin-treated cultures revealed that germ tube elongation of such cells became inhibited.

Protein biosynthesis. Trypan blue staining of cells taken from cultures described in the studies above showed that cells remained viable throughout the time course of the experiments. To further assess the effect of cerulenin and sodium butyrate on cell physiology, we determined the rate of protein biosynthesis in treated and untreated cells. These studies demonstrated that neither agent significantly affected the rate of protein biosynthesis for at least 140 min after the cultures were shifted to 37°C (Fig. 4).

Lipid biosynthesis. Because cerulenin is an inhibitor of fatty acid biosynthesis, it is likely that the agent inhibits *C. albicans* germination by interference with the biosynthesis of lipid moieties required for membrane biogenesis and function. However, evidence from other studies (12) and the fact that sodium butyrate-blocked germination cannot be reversed by fatty acids (see below) suggest that sodium butyrate-mediated inhibition of germination most likely occurs through an alternate mechanism; however, an indirect effect on lipid biosynthesis might result as a consequence of sodium butyrate treatment. To examine this issue further, we examined the rate of lipid biosynthesis in cerulenin- and sodium butyrate-treated *C. albicans* cultures stimulated to undergo morphogenesis (Fig. 5). As expected, cerulenin significantly inhibited the accumulation of lipids. In contrast, lipid biosynthesis in sodium butyrate-treated cultures was essentially identical to that of untreated controls.

Effect of palmitate on cerulenin- and sodium butyrate-promoted inhibition of germination. To confirm that cerulenin and sodium butyrate blocked germination by different mechanisms, we examined whether the addition of exogenous fatty acids might reverse the effect of either agent on morphogenesis. Supplementation of cerulenin-treated cultures with 0.01% palmitate (final concentration) completely restored the ability of cultures to undergo germination. In comparison, sodium butyrate-mediated inhibi-

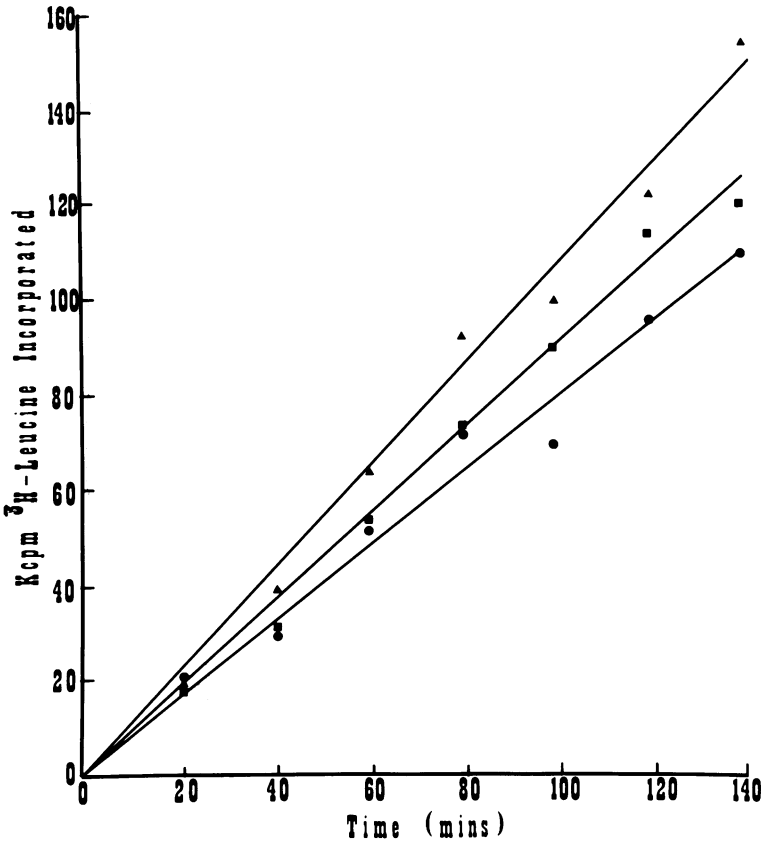

FIG. 4. Incorporation of [^{3}H]leucine into TCA-precipitable material in an untreated control (■) or in *C. albicans* cultures shifted to 37°C in the presence of cerulenin (●) or sodium butyrate (▲).

tion of germination could not be reversed by palmitate. Similarly, cultures treated with cerulenin, as well as with sodium butyrate, and supplemented with either 0.01 or 0.02% palmitate did not undergo germination.

Effect of cerulenin and sodium butyrate on chitin biosynthesis. We next investigated the effect of cerulenin and sodium butyrate on chitin biosynthesis during germination. Our evidence suggests that both agents abolish the increase of chitin synthesis that normally occurs upon the promotion of germination. Chitin biosynthesis in cerulenin-treated cells is only 54, 37, and 20% of the control values obtained between 20 and 40, 40 and 60, and 60 and 80 min postshift, respectively (Fig. 6). Likewise, chitin biosynthesis in sodium butyrate-treated cells was 38, 57, and 15% that of control cells over the same time intervals. Since protein biosynthesis during these time intervals is essentially identical in treated and untreated cultures (Fig. 4), the observed inhibition of chitin biosynthesis is not a reflection of a general depression in protein biosynthetic activity.

DISCUSSION

Cerulenin and sodium butyrate effectively blocked germination of *C. albicans*. Importantly, the concentrations used in this study did not adversely affect protein biosynthesis or cell viability during the time course of the experiments, and it is therefore unlikely that failure of cells to undergo morphogenesis was related merely to cell death.

The inhibitory effect of sodium butyrate on germ tube emergence was dependent on the time of exposure of *C. albicans* to the agent relative to the time of induction of germination. In this regard, our results suggest that sodium butyrate blocks the biosynthesis or assembly of some component required for germination and that this requirement was satisfied by 45 to 60 min after the stimulation of germination. In light of evidence obtained in other systems (1, 12), sodium butyrate might interfere with cytoskeletal assembly which in turn could prevent the polarization of cell wall biogenesis that accompanies morphogenesis. Alternatively, sodium butyrate-

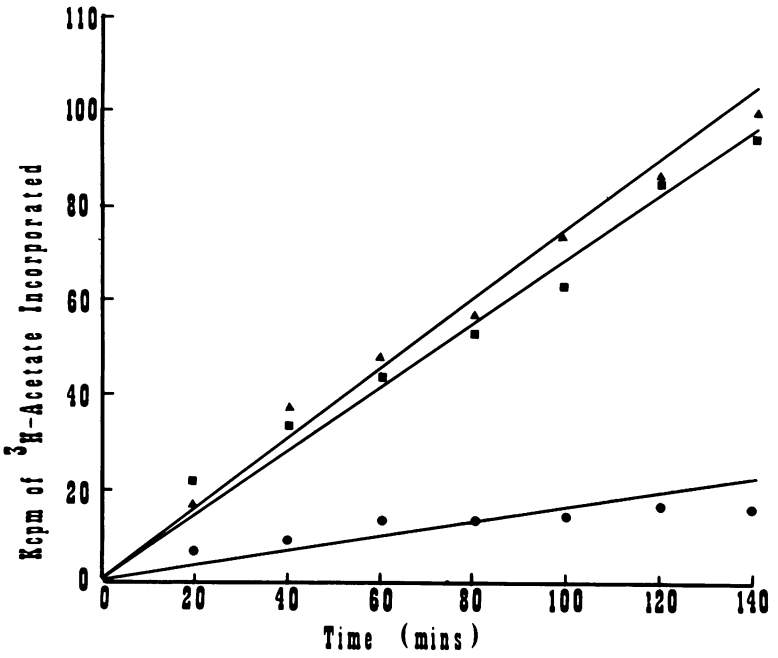

FIG. 5. Incorporation of [^{3}H]acetate into hot methanol-chloroform-extractable material in untreated controls (■) or in *C. albicans* cultures shifted to 37°C in the presence of cerulenin (●) or sodium butyrate (▲).

induced hyperacetylation of histones (20) may interfere with the differential regulation of certain key gene products necessary for hyphal growth to proceed. Certainly, other possibilities can be envisioned as well, and these issues are currently under investigation.

Similarly, *C. albicans* germination increased dramatically when cerulenin was added at times after incubation of cultures at 37°C. In this case, it is likely that when cultures were supplemented with cerulenin at later times relative to the shift in incubation temperature, existing lipids synthesized before drug addition may have been sufficient to allow germ tube emergence to begin. However, with the depletion of available lipids, germ tube elongation slowed, resulting in the stunted germlings observed in these experiments.

We have also demonstrated that, whereas cerulenin presumably blocks germ tube emergence by inhibition of lipid biosynthesis, sodium butyrate treatment has no effect on the rate of lipid biosynthesis, although in this case qualitative or quantitative differences in the biosynthesis of individual lipids have not been ruled out. In addition, inhibition of germination by cerulenin can be overcome by palmitate, but supplementation of sodium butyrate or sodium butyrate- and cerulenin-treated cultures with palmitate failed to promote germination. These findings suggest that, although lipid biosynthesis is necessary during *C. albicans* germination, fulfillment of this requirement does not ensure that morphogenesis will occur. The results also indicated that use of both agents will allow a distinction to be made between events coupled to membrane or membrane-bound organelle biogenesis that are directly related to germination and events that might be affected by membrane integrity but that are not essential for hyphal development.

In this regard, we have examined the effect of cerulenin and sodium butyrate on chitin biosynthesis during germination. The reasons for this choice were severalfold: (i) chitin synthesis is differentially regulated during germination (3, 6) and may be a critical event in the process, (ii) chitin synthase is associated with the plasma membrane and may require phospholipids or sterols for maximal activity (3, 9, 19), and (iii) the study of chitin synthase and chitin deposition allows an examination of the interrelationship between membrane and cell wall activity during morphogenesis. Our evidence indicates that both agents inhibit the increase in chitin biosynthesis that normally accompanies germ tube emergence. This result further underscores the potential importance of chitin biosynthesis in germination, since the agents blocked germination by different mechanisms. It is also possible that the inhibitory effect of each agent on chitin biosynthesis occurs by different routes. For ex-

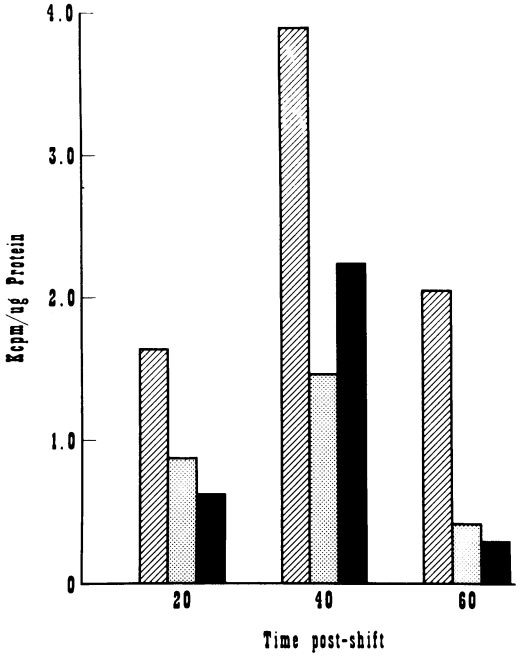

FIG. 6. Effect of cerulenin and sodium butyrate on chitin biosynthesis. Either cerulenin or sodium butyrate was added to cultures 30 min before induction of germination. An identical but untreated culture was maintained as a control. Cultures were shifted to 37°C; samples were removed after 20, 40, and 60 min; and [^{3}H]*N*-acetylglucosamine was added to the cultures at that time. After a 20-min pulse, cells were harvested and chitin was isolated. Chitin biosynthesis was quantitated and the results were normalized to total protein. Dotted bars, cerulenin-treated culture; solid bars, sodium butyrate-treated culture; cross-hatched bars, untreated culture.

ample, by perturbing the normal environment, cerulenin may interfere with the phospholipid-enzyme interactions necessary for maximal enzyme activity. The demonstration by others of lipid components in chitosomes (10) suggests that this could be an explanation. In contrast, sodium butyrate does not interefere with lipid biosynthesis and presumably would not inhibit chitin biosynthesis in this fashion, but rather might influence zymogen activation, etc. Thus, exploitation of both agents may provide a way to study the regulation of processes leading to chitin synthase induction. Likewise, a study of other membrane-associated enzymes (glucan and mannan synthases, etc. [5, 15]) and functions (transport of cell wall polymers etc. [8, 21]), as well as investigations concerning the importance of these parameters in *C. albicans* germination, should be facilitated by use of cerulenin and sodium butyrate as inhibitors of *C. albicans* morphogenesis.

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LITERATURE CITED

1. **Altenburg, B. C., D. P. Via, and S. H. Steinei.** 1976. Modification of the phenotype of murine sarcoma virus-transformed cells by sodium butyrate. Exp. Cell Res. **102**:223–231.
2. **Brambl, R., H. Wenzler, and M. Josephson.** 1978. Mitochondrial biogenesis during fungal spore germination: effects of the antilipogenic antibiotic cerulenin upon *Botryodiplodia* spores. J. Bacteriol. **135**:311–317.
3. **Braun, P. C., and R. A. Calderone.** 1978. Chitin synthesis in *Candida albicans*: comparison of yeast and hyphal forms. J. Bacteriol. **135**:1472–1477.
4. **Chance, K., S. Hemmingsen, and G. Weeks.** 1976. Effect of cerulenin on the growth and differentiation of *Dictyostelium discoideum*. J. Bacteriol. **128**:21–27.
5. **Chattaway, F. W., M. R. Holmes, and A. J. E. Barlow.** 1968. Cell wall composition of the mycelial and blastospore forms of *Candida*. J. Gen. Microbiol. **123**:233–240.
6. **Chiew, Y. Y., M. G. Shepherd, and P. A. Sullivan.** 1980. Regulation of chitin-synthesis during germ tube formation in *Candida albicans*. Arch. Microbiol. **125**:97–104.
7. **Contreras, I., A. Weissborn, K. Anemiya, J. Mansour, S. Henry, R. Bender, and L. Shapiro.** 1980. The effect of termination of membrane phospholipid synthesis on cell-dependent events in *Caulobacter*. J. Mol. Biol. **138**:401–409.
8. **Dow, J. M., R. R. Carreon, and V. D. Villa.** 1981. Role of membranes of mycelial *Mucor rouxii* in synthesis and secretion of cell wall matrix polymers. J. Bacteriol. **145**:272–279.
9. **Duran, A., B. Bowers, and E. Cabib.** 1975. Chitin synthetase zymogen is attached to the yeast plasma membrane. Proc. Natl. Acad. Sci. U.S.A. **72**:3952–3955.
10. **Hernandez, J., E. Lopez-Romero, J. Cerbon, and J. Ruiz-Herrera.** 1981. Lipid analysis of chitosomes, chitin-synthesizing microvesicles from *Mucor rouxii*. Exp. Mycol. **5**:349–356.
11. **Ito, E. T., R. L. Cihlar, and C. B. Inderlied.** 1982. Lipid synthesis during morphogenesis of *Mucor racemosus*. J. Bacteriol. **152**:880–887.
12. **Kruh, J.** 1982. Effects of sodium butyrate, a new pharmacological agent, on cells in cutlure. Mol. Cell. Biochem. **42**:65–82.
13. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
14. **Manning, M., and T. G. Mitchell.** 1980. Strain variation and morphogenesis of yeast- and mycelial-phase *Candida albicans* in low-sulfate, synthetic medium. J. Bacteriol. **142**:714–179.
15. **Marriott, M. S.** 1977. Mannan-protein location and biosynthesis in plasma membranes from the yeast form of *Candida albicans*. J. Gen. Microbiol. **103**:51–59.
16. **Nickerson, K. W., and E. Leastman.** 1978. Cerulenin inhibition of spore germination in *Rhizopus stolonifer*. Exp. Mycol. **2**:26–31.
17. **Odds, F. C.** 1979. *Candida* and candidiosis. University Park Press, Baltimore, Md.
18. **Ōmura, S.** 1976. The antibiotic cerulenin, a novel tool for biochemistry as an inhibitor of fatty acid synthesis. Bacteriol. Rev. **40**:681–697.
19. **Pesti, M., J. M. Cambell, and J. F. Peberdy.** 1981. Alteration of ergosterol content and chitin synthase activity in

Candida albicans. Curr. Top. Microbiol. Immunol. **5**:187–190.

20. **Riggs, M. G., R. G. Whittaker, J. R. Neumann, and V. M. Ingram.** 1977. n-Butyrate causes histone modification in HeLa and Friend erythroleukaemia cells. Nature (London) **268**:462–464.
21. **Singh, M., A. Sayakumar, and R. Prasad.** 1979. The effect of altered ergosterol content on the transport of various amino acids in *Candida albicans*. Biochim. Biophys. Acta **555**:42–44.