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Mitochondrial Biogenesis During Fungal Spore Germination: Effects of the Antilipogenic Antibiotic Cerulenin upon *Botryodiplodia* Spores

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Germination of spores of the fungus *Botryodiplodia theobromae* was inhibited by the antilipogenic antibiotic cerulenin. The spores remained viable in the presence of the antibiotic, however, and after prolonged incubation they were able to overcome the inhibition. Cerulenin inhibition of germination was reversed by Tween 40 and Tween 60 (derivatives of palmitate and stearate, respectively), but not by representatives of a range of free fatty acids or their soaps. Cerulenin abolished incorporation of [^{14}C]acetate into sterols and triglycerides and reduced its incorporation into fatty acids by 69%. Cyanide-sensitive oxygen consumption by spores incubated in the presence of cerulenin was greatly reduced throughout germination, and the activity of cytochrome *c* oxidase was no more than 13% of the activity in untreated spores, even after prolonged incubation. However, low-temperature difference spectra of mitochondrial extracts showed that the cerulenin-treated spores accumulated a threefold excess of cytochrome *a*, whereas the cellular concentrations of cytochromes *c* and *b* were identical to those of untreated spores. Cerulenin treatment sharply reduced the rates of whole spore protein and RNA synthesis. Cerulenin had no effects upon mitochondrial morphology which could be discerned with an electron microscope.

During the germination of fungal spores, the resumption of certain metabolic activities may depend upon assembly and function of new cell membrane components and structures. The activity of a membrane structure, such as the mitochondrial electron transport system, could require not only new protein synthesis during germination, but also new lipid synthesis and elaboration of a suitable lipid environment for the membrane-associated enzyme polypeptides. It seems likely, therefore, that experimental inhibition of lipid synthesis in germinating spores would provide useful insight into the role of membrane lipids in assembly or modification of the mitochondrial respiratory system as the cells undergo a transition from metabolic quiescence to rapid growth.

The antibiotic cerulenin interferes with fatty acid biosynthesis (apparently through inhibition of the fatty acid synthetases) in a wide range of organisms, including bacteria, cellular slime molds, yeasts, and mycelial fungi (for a detailed review, see reference 16). In some cases cerulenin simultaneously inhibits sterol biosynthesis (15). This antibiotic provides a novel, useful means of examining the general role of lipid synthesis (and presumably membrane lipid synthesis) in the biogenesis and function of cellular membranes and organelles.

Our previous studies with the spores of *Botryodiplodia theobromae* (a homothallic ascomycete) demonstrated that the dormant conidia contain some, but not all, components of a mitochondrial aerobic respiratory system. Cytochrome *a*, heme *a*, and cytochrome *c* oxidase activity are absent in the dormant spore mitochondria (4, 6). Products of cytoplasmic ribosomes (but not those of mitochondrial ribosomes) are required for elaboration and function of the respiratory system in the germinating spores (3-5).

Although these earlier studies with *B. theobromae* spores primarily have sought to understand mitochondrial assembly in terms of differential ribosome product requirements, it is likely that the lipid composition of the mitochondrial membranes also is involved in mitochondrial assembly and function. In the present study we describe the consequences of cerulenin treatment upon germinating spores and upon the elaboration of spore mitochondrial respiratory activity.

MATERIALS AND METHODS

The *B. theobromae* spores were produced and germinated under conditions described previously (3). The chemically defined incubation medium contained 0.5% (vol/vol) Tween 80 and 3 μg of ergocalciferol per

ml as part of a vitamin supplement. In all germination assays, at least 300 spores were scored at each measurement.

Sodium [1,2-^{14}C]acetate (specific activity, 54 mCi/mmol), L-[*U*-^{14}C]leucine (specific activity, 314 mCi/mmol), and [2-^{14}C]uracil (specific activity, 54.8 mCi/mmol) were obtained from New England Nuclear Corp., Boston, Mass. Free fatty acids and Tweens 40, 60, and 80 were purchased from U.S. Biochemical Corp., Cleveland, Ohio, and Sigma Chemical Co., St. Louis, Mo., respectively. Cerulenin was obtained from Makor Chemicals, Jerusalem, Israel; stock solutions of 20 mg of 95% ethanol per ml were prepared and stored at −20°C.

The methods for measurement of spore oxygen uptake with an oxygen electrode have been described previously (3), as have the methods for measurement of total spore protein and RNA syntheses by pulse-labeling techniques (7).

Extraction of the mitochondrial cytochrome fraction with sodium cholate and $(NH_4)_2SO_4$ and low-temperature (77°K) difference spectrophotometry of the extracted cytochromes were performed as reported previously (6). Relative concentrations of the cytochromes were calculated using the room-temperature molar extinction coefficients (cytochrome *c*, 19.0 mM^{-1} cm^{-1} [9]; cytochrome *b*, 20.0 mM^{-1} cm^{-1} [10]; cytochrome *a*, 24.0 mM^{-1} cm^{-1} [18]) and the absorbance differences between α-band absorption maxima and reference wavelengths for each of the cytochromes (cytochrome *c*, 549 to 537 nm; cytochrome *b*, 559 to 569 nm; cytochrome *a*, 604 to 619 nm). The enhancement factors of the cytochrome absorption bands caused by the liquid nitrogen temperature are unknown. Therefore, although determinations of the absolute concentrations of the cytochromes are quantitatively incorrect, the values do permit calculations of the molar ratios of cytochrome concentrations between the cerulenin-treated and untreated spore mitochondrial extracts. In addition, areas under the cytochrome α-band absorption peaks were measured with a photometric area meter (type 5; Hayashi Denko Co., Tokyo, Japan) to test the reliability of the relative concentration determinations. Procedures have been published elsewhere (4) for extraction and analysis of the cytochrome *c* oxidase fraction from the mitochondria of these spores.

For measurement of lipid synthesis in the presence or absence of cerulenin (50 μg/ml), the spores were incubated for 240 min with [^{14}C]acetate (0.05 μCi/ml in 1 mg of spore suspension per ml). The spores were harvested by filtration, washed with water, and disrupted in a CO_2-cooled Braun MSK mechanical homogenizer (45 s, 4,000 rpm), using a ratio of 200 mg of spores (fresh weight) to 10 ml of chloroform-methanol (2:1, vol/vol) to 27 g of 1-mm glass beads. An additional 20 ml of chloroform-methanol was added to the homogenized spores, and the mixtures were left overnight at room temperature in tightly stoppered flasks. Lipids were partitioned with an aqueous wash procedure (11), and, after evaporation of the chloroform solvent, the lipid residues were dissolved in hexane. Samples of hexane-dissolved lipids from control and cerulenin-treated spores were counted in a liquid scintillation spectrometer to give an estimate of acetate incorporation into the chloroform-soluble fraction. Lipid classes were separated by thin-layer chromatography, using 0.25-mm-layer Silica Gel 60 precoated glass plates (5 by 20 cm; E. Merck AG, Darmstadt, Germany). Samples were applied as spots to the plates which were developed in paper-lined glass chambers, using a solvent system of hexane-diethyl ether-glacial acetic acid (85:15:1, vol/vol/vol). A standard lipid mixture containing cholesteryl oleate, methyl oleate, triolein, oleic acid, and cholesterol was co-chromatographed on each plate, and the chromatographic separation of the standard lipids was observed by spraying the plates with 18 N H_2SO_4 and charring for 5 min in a 100°C oven. We chromatographed the labeled spore extracts on duplicate plates which were not charred, removed the zones of the silica gel which corresponded to the zones of the lipid standards, and determined the radioactivity by liquid scintillation spectrometry.

RESULTS

The standard defined medium used in our previous studies with *B. theobromae* contains 0.5% Tween 80; we have found that, for unexplained reasons, this supplement increases the rate and synchrony of spore germination and accelerates several metabolic activities (unpublished data). At the outset of this study, we wished to establish whether the Tween 80 would decrease the inhibitory effect of cerulenin in comparison with another medium containing only 2% glucose and 0.5% yeast extract. However, we learned that although the spores incubated in the defined medium were readily inhibited by lower concentrations of cerulenin (25 and 50 μg/ml), the same spores in the medium containing yeast extract were only slightly inhibited with the same concentrations of the drug. Apparently there were components of the yeast extract which reversed or blocked the inhibitory effect of cerulenin; these results also suggested that the inhibition of germination by the drug could not be reversed by the oleate component of Tween 80 (as shown in a later experiment). In subsequent experiments, the incubation media contained 0.5% Tween 80.

The effect of different concentrations of cerulenin upon spore germination was measured (Fig. 1). Cerulenin almost completely inhibited spore germination at 240 min at the higher drug concentrations, whereas decreasing concentrations permitted correspondingly more germination. Although spore germination at 240 min was inhibited by 50 μg of cerulenin per ml, the germination percentage of these same spores by 480 min (8-h total) was about 90% (although the germ tubes were very short). Such results indicate that, at this concentration, cerulenin actually retards germination, instead of abolishing it, and they show that the spores eventually overcome the effects of the antibiotic sufficiently

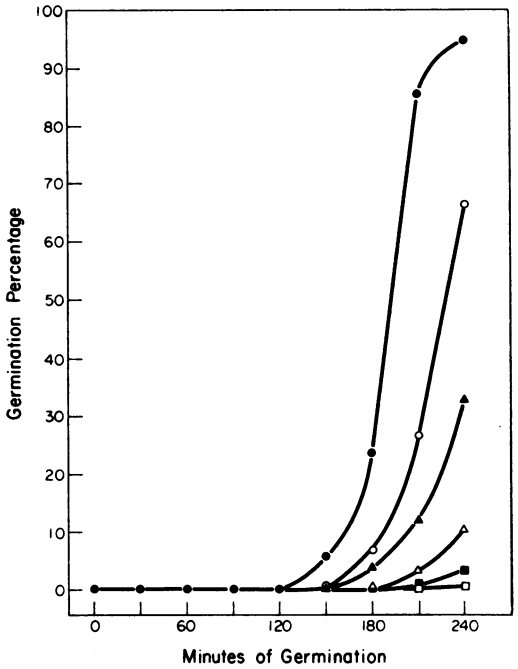

FIG. 1. *Effects of different concentrations of cerulenin upon rates of spore germination. Symbols: ●, untreated control; ○, 5 μg/ml; ▲, 10 μg/ml; △, 25 μg/ml; ■, 50 μg/ml; and □, 100 μg/ml.*

to allow germination, although all metabolic activities are not necessarily restored to normal (as described below).

In two yeast genera (2, 14) and in *Dictyostelium* (8) the inhibitory effect of cerulenin is at least partially reversed by the addition of certain fatty acid supplements. However, we found that a series of free fatty acids (elaidic, linoleic, linolenic, myristic, oleic, palmitic, and stearic acids) at either 250 or 500 μM had no reversal effect upon the spores inhibited with cerulenin (50 μg/ml). Sodium soaps of these fatty acids at 250 or 500 μM also did not reverse the cerulenin inhibition of these *Botryodiplodia* spores. Control experiments showed that the fatty acids or their solvent (ethanol) had no adverse effect upon spore germination. Subsequently, we tested the effects of Tween 40, Tween 60, and Tween 80 (polyoxyethylene sorbitan derivatives of palmitate, stearate, and oleate, respectively) and found that, whereas the Tween 80 (2%, vol/vol) permitted only about 20% germination at 240 min in the presence of 50 μg of cerulenin per ml, Tween 60 (2%, vol/vol) and Tween 40 (2%, vol/vol) permitted about 60 and 70% germination, respectively (Fig. 2). About 90% of these Tween-treated spores germinated by 480 min. Therefore, although cerulenin inhibition of *Botryodiplodia* spore germination could not be reversed directly by fatty acid supplementation, the inhibition of germination was relieved by Tween 40 and Tween 60, which are sources of palmitic and stearic acids.

To measure the effect of cerulenin upon lipid synthesis, we labeled the spores with [^{14}C]acetate for 240 min in the presence or absence of cerulenin (50 μg/ml). The lipid fraction was extracted and chromatographed on thin-layer plates of silica gel along with a set of lipid standards; the zones of the sample plates which corresponded to the zones of the standards were analyzed for radioactivity. The untreated spores consumed about 12% of the exogenous [^{14}C]acetate, and the cerulenin-treated spores consumed about 3% of the precursor. The distributions of label from [^{14}C]acetate into the lipid classes of the treated and untreated spores are compared in Table 1. The syntheses of compounds which chromatographed congruently with sterols, sterol esters, and triglycerides were almost abolished, whereas the incorporation of label into fatty acids was less severely inhibited. The cerulenin-treated spores synthesized a large quantity of an unidentified substance which chro-

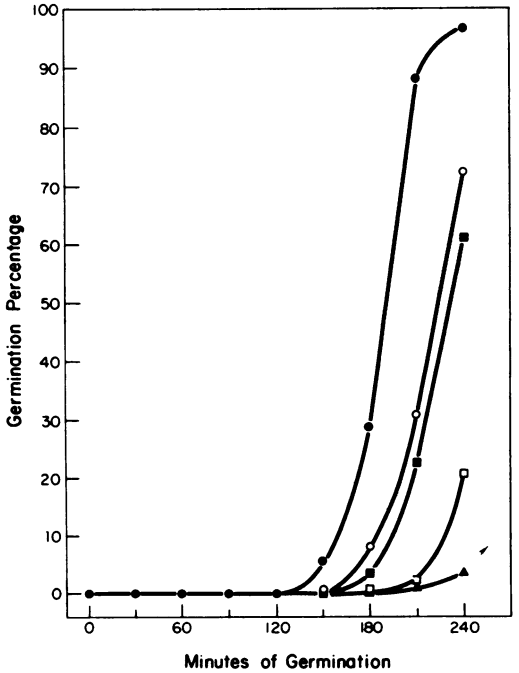

FIG. 2. *Reversal effects of Tween 80 (□), Tween 60 (■), and Tween 40 (○) upon spores incubated in the presence of 50 μg of cerulenin per ml. Germination rates also are shown for spores incubated in the absence (●) or presence (▲) of cerulenin without supplementary Tweens at 2% concentrations.*

TABLE 1. *Thin-layer chromatographic distribution of [^{14}C]acetate radioactivity among several cell fractions of spores labeled in the presence or absence of cerulenin (50 μg/ml)*

Fraction[a]	Incorporation of [^{14}C]acetate per 200 mg of spores		
	Control (dpm)	Cerulenin treated (dpm)	Inhibition (%)
Whole spores[b]	2,621,000	632,000	75.9
Chloroform-methanol soluble[c]	519,480	63,060	87.9
Origin	129,180	13,020	89.9
Sterol	28,740	540	98.1
Fatty acid	31,920	10,020	68.6
Triglyceride	204,240	3,180	98.4
Fatty acid methyl ester	0	23,880	0
Sterol ester	12,360	0	100

[a] The lipid fractions in this column represent zones of the chromatographic plate which migrate congruently with authentic samples of cholesterol, oleic acid, triolein, methyl oleate, and cholesteryl oleate.

[b] A 5-ml sample of each spore suspension was withdrawn at 240 min, the suspension was made 5% with respect to trichloroacetic acid, and the spores were transferred immediately to a glass fiber filter disk, washed with 50 ml of cold 5% trichloroacetic acid, and dried for liquid scintillation spectrometry.

[c] Measured after removal of the chloroform-methanol solvent.

matographed with the fatty acid methyl ester, whereas the untreated control spores synthesized none of this compound. The results of this experiment show clearly that cerulenin does inhibit synthesis of lipids from acetate (as in other organisms) and that both fatty acid and sterol syntheses in *B. theobromae* are affected by this antibiotic.

Measurements of oxygen uptake rates were made during germination to determine the effect of cerulenin treatment upon development of the spore aerobic respiratory competence. The untreated control spores showed a biphasic curve of cyanide-sensitive oxygen uptake rates (Fig. 3), with an acceleration of uptake at about 150 min, which we described previously (3). The oxygen consumption of the cerulenin-treated (50 μg/ml) spores was sharply reduced during the first 150 min of incubation; after this point the respiratory rates slowly accelerated, so that by 300 min the treated spores consumed oxygen at about 20% of the rate of the control spores. The oxygen uptake of the cerulenin-treated spores was cyanide sensitive and salicylhydroxamic acid insensitive (data not shown).

The inhibition of oxygen consumption by cerulenin suggested that development of the mitochondrial respiratory system was disrupted, and we examined this possibility directly by low-temperature difference spectrophotometry of the mitochondrial cytochromes. A cytochrome fraction was prepared from 8-h untreated spores and 8-h cerulenin-treated (50 μg/ml) spores. We chose the 8-h time point because our previous work (6) showed that, by then, the spores normally should have elaborated sufficient quantities of all the respiratory cytochromes to yield strong absorption bands. Figure 4 shows the cytochrome patterns of the cerulenin-treated and the untreated spores recorded at 77°K. It is striking that the cerulenin-treated and control spore mitochondrial extracts contained the same quantities of cytochrome *c* (549 nm) and cytochrome *b* (559 nm), but that there was a much greater quantity of cytochrome *a* (604 nm) in the extract of cerulenin-treated spores. Calculations of the quantities of cytochromes *c* and *b* in the mitochondrial extracts (after compensating for differences in protein concentration) showed that the molar ratios of these two cytochromes between the treated and untreated spores were near unity. In contrast, the ratio of cytochrome *a* in the cerulenin-treated versus the control spores was 3:1. Measurements with a photomet-

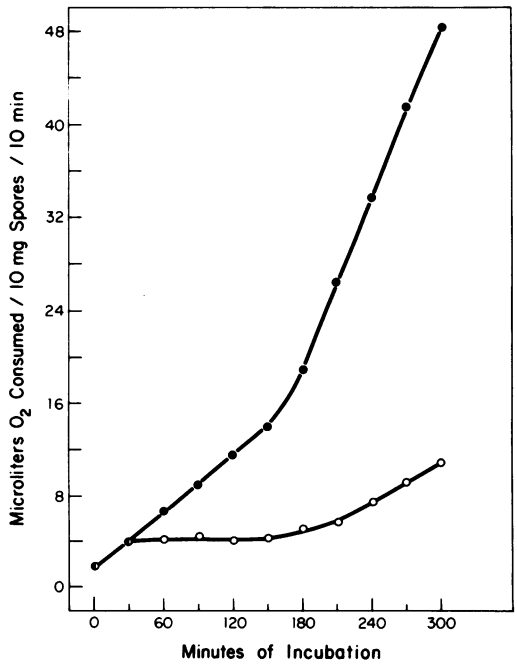

FIG. 3. *Rates of cyanide-sensitive oxygen consumption by control spores incubated in the absence of cerulenin (●) and by cerulenin-treated spores (○). These data are corrected for the low amount of oxygen uptake (2 μl/10 mg of spores per 10 min) which occurs in the presence of 0.5 mM NaCN and which is constant during this incubation period.*

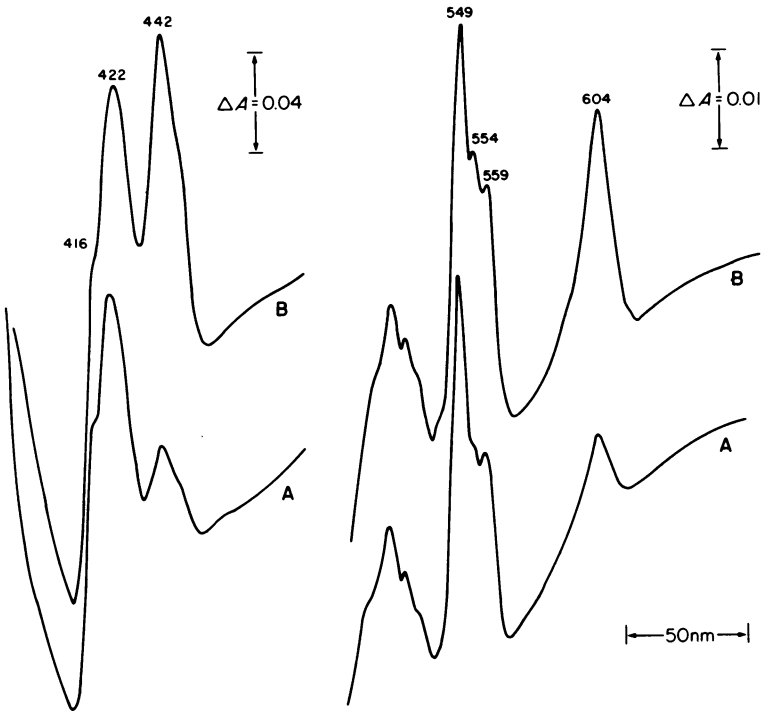

FIG. 4. *Oxidized versus reduced cytochrome absorption difference spectra (77°K) of mitochondrial cytochrome fractions isolated from untreated, 8-h control spores (trace A) or from cerulenin-treated, 8-h spores (trace B). The quantities of protein in the 1-ml cells of the cuvette were 1.455 mg (control) and 1.914 mg (cerulenin treated). ΔA, Change in absorbancy.*

ric area meter confirmed that the ratios of absorption peak areas of cytochromes *b* + *c* were near unity in the treated and untreated spores, whereas the cytochrome *a* peak area was three times larger in the cerulenin-treated spores (after compensation for differences in protein concentration). Differences in the amounts of cytochrome *a* in the two types of mitochondria also are reflected in the Soret peaks (442 nm) of the two mitochondrial extract spectra. The cerulenin-treated spores had attained about 90% germination by 8 h. Spores germinated in the presence of cerulenin and Tween 40 had a normal complement of mitochondrial cytochromes.

We observed previously that at 240 min of incubation cerulenin treatment had caused a sharp reduction in the amount of cytochrome *c* oxidase activity which could be extracted from the spores (4). Because of the finding of an apparent excess of cytochrome *a* in the cerulenin-treated spore mitochondria, we reexamined the elaboration of the cytochrome *c* oxidase during germination and measured the activity of the enzyme through 8 h of growth. The data in Fig. 5 confirm our earlier observation that the antibiotic (50 μg/ml) severely inhibits development of the enzyme activity in the first 240 min

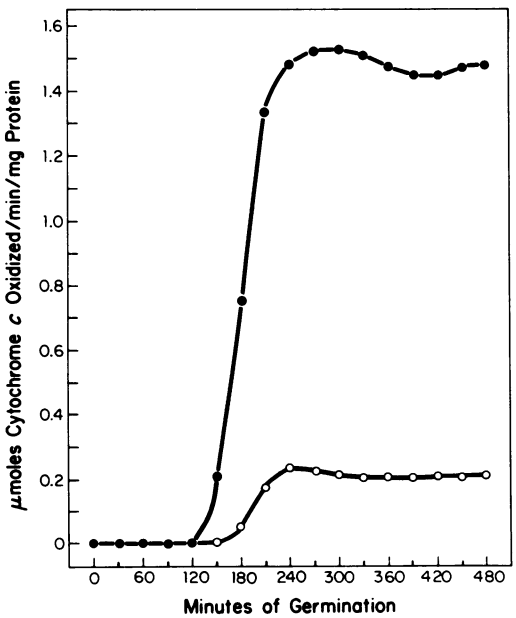

FIG. 5. *Kinetics of development of cytochrome c oxidase activity during incubation of untreated control spores* (●) *and cerulenin-treated spores* (○).

of incubation. However, this experiment also shows that at 8 h of growth the cerulenin-treated spores contained only about 13% of the cytochrome *c* oxidase activity of the untreated spores. Therefore, the cerulenin treatment caused the spores to accumulate a threefold excess of cytochrome *a* while simultaneously causing a sevenfold reduction in the cytochrome *c* oxidase activity.

Whole spore protein and RNA syntheses were measured by pulse-labeling techniques, and the kinetics of incorporation of [^{14}C]leucine and [^{14}C]uracil into acid-insoluble fractions are shown in Fig. 6. As shown previously (3, 7), the spore protein synthesis began in the untreated control spores immediately upon inoculation into the incubation medium; initiation of [^{14}C]-uracil incorporation was delayed until about 60 min (7), and the early [^{14}C]leucine incorporation depended upon translation of a latent mRNA (7).

The labeled leucine and uracil were incorporated almost entirely into the cellular fractions chemically identified as protein and RNA. Previously published studies (7, 12) with these spores indicate that these patterns of protein and RNA syntheses are not likely affected by endogenous precursor pools or by cellular impermeability to the exogenous, labeled precursors. We found that incorporations of [^{14}C]leucine and [^{14}C]uracil into protein and RNA were sharply reduced as a consequence of the 50-μg/ml cerulenin treatment (Fig. 6). Early in germination, the cerulenin-treated spores incorporated [^{14}C]leucine at rates of about 30% of those of the untreated spores, and at 240 min the drug-treated spores incorporated the [^{14}C]leucine at about 10% of the rate of the untreated spores. The normal initiation and increase in rates of [^{14}C]uracil incorporation after 60 min were delayed until after 150 min by the cerulenin treatment, and, although the rates of [^{14}C]uracil incorporation accelerated in relation to the [^{14}C]leucine incorporation, at 240 min the [^{14}C]-uracil was incorporated at only about 15% of the rate in the untreated spores. These inhibitions of protein and RNA syntheses by cerulenin were almost eliminated if Tween 40 was included in the germination medium.

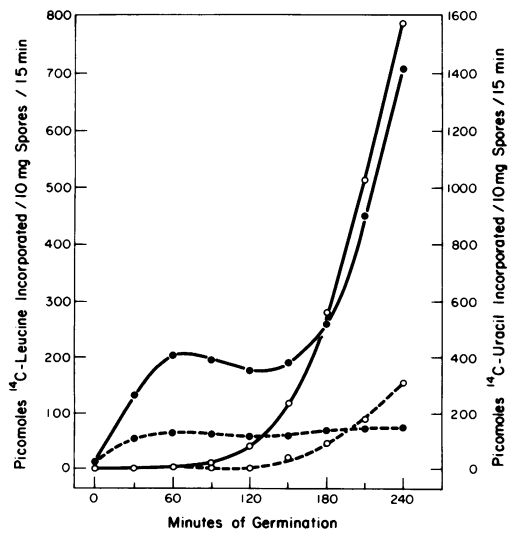

FIG. 6. *Rates of spore protein and RNA syntheses measured by pulse-label incorporation of [^{14}C]leucine (●) and [^{14}C]uracil (○) in the presence (- - - -) or absence (——) of cerulenin. The correction for zero-time incorporation in this graph was less than 6 pmol/assay.*

DISCUSSION

An earlier report from this laboratory provided the first evidence that fungal spore germination could be inhibited by the antibiotic cerulenin (4). In the present study we have shown directly that this drug inhibits lipid synthesis in the germinating spores of *B. theobromae,* that one of the consequences of cerulenin treatment is an inhibition of function of the developing mitochondrial respiratory system, and that the effect of cerulenin upon spore germination may be reversed by inclusion of certain saturated fatty acid supplements in the spore incubation medium. We conclude that lipid synthesis is essential for *B. theobromae* spore germination and probably is required for membrane assembly.

This reversible inhibition of fatty acid synthesis in germinating spores sharply reduced the rates of cyanide-sensitive oxygen consumption as well as the activity of cytochrome *c* oxidase. Simultaneously, however, the cerulenin-treated spores accumulated a threefold excess of cytochrome *a*, while the relative concentrations of cytochromes *b* and *c* were unchanged. Experiments are now in progress to determine the effects of this antibiotic upon the assembly of cytochrome *c* oxidase in these spores and to establish how cellular synthesis or accumulation of the individual enzyme polypeptide subunits may be affected by cerulenin.

In addition to the effects upon the spore respiratory system, cerulenin treatment sharply reduced the rates of whole spore protein and RNA syntheses throughout the 240-min germination sequence. Although these inhibitions could be due to an unidentified side effect of cerulenin, it also is possible that the antibiotic interferes with the synthesis or metabolism of a membrane lipid required for the protein synthesis which begins early in germination before initiation of RNA synthesis (7, 12). This inhibition of protein syn-

thesis could be accomplished indirectly through inhibition of energy-generating functions, as indicated by the effects of cerulenin upon spore respiration.

We prepared mitochondrial fractions from dormant, germinated, and cerulenin-treated spores, fixed them with glutaraldehyde, and stained them with OsO_4 for thin-section observation in an electron microscope (at ×28,000 magnification). There were no observable morphological differences between mitochondria of the treated and untreated spores (data not shown). Although mitochondria from the dormant spores frequently appeared to be smaller in diameter, the numbers of cristae per unit volume appeared to be very similar in mitochondria from all three spore preparations. These results indicate that cerulenin has no effect upon spore mitochondrial morphology which can be discerned with an electron microscope.

In this study, as well as in other studies which have employed cerulenin, an assumption is made that the drug inhibits de novo synthesis of lipids required for membrane biogenesis. Although no direct evidence has been available to support this assumption, our measurements of the effects of cerulenin treatment upon the spore mitochondrial respiratory system (whose functions are sensitive to altered membrane lipid composition [1, 13, 17]) show that cerulenin does disrupt membrane assembly and function. These experiments also show that lipid synthesis for new membrane organization (in addition to protein synthesis) is essential for certain cellular developmental transitions, such as that exemplified by fungal spore germination.

ACKNOWLEDGMENTS

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ADDENDUM IN PROOF

While this paper was in press, another appeared (K. W. Nickerson and E. Leastman, Exp. Mycol. **2**:26–31, 1978) which describes several effects of cerulenin upon germination of *Rhizopus stolonifer* spores.

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