RESPIRATION OF BASIDIOSPORES OF
SCHIZOPHYLLUM COMMUNE

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ABSTRACT

NIEDERPRUEM, DONALD J. (Indiana University Medical Center, Indianapolis). Respiration of basidiospores of *Schizophyllum commune*. J. Bacteriol. **88**:210–215. 1964.—The aerobic metabolism of basidiospores of the wood-rotting mushroom *Schizophyllum commune* was investigated by use of manometric techniques and specific respiratory poisons. Basal respiration was stimulated markedly by the uncoupling agent 2,4-dinitrophenol. This effect was pH-dependent and was sensitive to antimycin A. A positive periodic acid-Schiff reaction and a respiratory quotient (CO_2/O_2) of near unity pointed to carbohydrate as the endogenous substrate. Oxygen consumption was increased by sucrose, certain hexoses, D-xylose, acetate, and ethanol. Oxidative assimilation was evident with D-glucose and acetate. Glucose oxidation was inhibited by cyanide, azide, antimycin A, Atabrine, and phenylmercuric acetate. These data implicate cytochrome oxidase, b- and c-type cytochromes, flavoprotein, and essential sulfhydryl groups in basidiospore respiration.

A variety of complex, resistant forms (e.g., bacterial spores and microcysts, and ascospores of fungi) characterize certain inhabitants of the microbial world. Usually, the metabolism of these resistant stages is barely perceptible and, more specifically, respiration is extremely low. Whereas vegetative cells of aerobic sporeforming bacteria (Smith, 1954) and microcyst-forming myxobacteria (Dworkin and Niederpruem, 1964) contain a complete and functional cytochrome system, the bacterial spore appears to lack most of these respiratory pigments (Keilin and Hartree, 1947; Spencer and Powell, 1952; Doi and Halvorson, 1961). In contrast, the microcyst of *Myxococcus xanthus* contains a complete cytochrome system, although very little respiratory activity can be demonstrated with the intact microcyst (Dworkin and Niederpruem, 1964). Ascospores of fungi and, in particular, *Neurospora tetrasperma* remain dormant until appropriate heat or chemical activation, after which a striking increase in respiratory activity occurs (Sussman, 1961). Basidiospore metabolism, on the other hand, is only poorly understood, and there is no information concerning the physiology of basidiospores of the wood-rotting mushroom *Schizophyllum commune*. A functional tricarboxylic acid cycle was demonstrated in mycelium of *S. commune* (Wessels, 1959), and terminal respiration is believed to be mediated by a series of hydrogen (electron) carriers, including pyridine nucleotide, flavoprotein, cytochromes of the a, b, and c types, and cytochrome oxidase (Niederpruem and Hackett, 1961; Dowler, Shaw, and Gottlieb, 1963). This poses the following questions. Are the basidiospores of *S. commune* capable of aerobic metabolism? What is the nature of the respiratory system in these reproductive units? This report offers data, obtained largely through manometric studies of intact basidiospore respiration and the employment of specific metabolic poisons, which indicate that a functional respiratory chain similar to that of vegetative mycelium is present in basidiospores of *S. commune*.

MATERIALS AND METHODS

Culture conditions. *S. commune* Fr. was cultured and mated on a minimal medium which contained (per liter of distilled water): D-glucose, 20 g; asparagine (Difco), 2 g; thiamine hydrochloride, 100 µg; KH_2PO_4, 0.46 g; K_2HPO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; and Noble Agar, 20 g. Basidiospores were collected from the cross, 699 A[41]B[41] × 845 A[51]B[51], which involves two good-fruiting strains of *S. commune*.

Sporulating fruits were obtained from crosses incubated at 25 C (±3 C) in the light (fluorescent lamp, 175 ft-c, ±50). Because dry weather causes a divergence of the gill plates and, hence, decreases spore emission of *S. commune* (Buller, 1909), the young fruits were transferred to a moist chamber to ensure maximal basidiospore yields.

Cytochemical methods. Cytochemical reactions

were performed as outlined by Zalokar (1959) for *Neurospora* with minor modifications. Smears of basidiospores were fixed with either methanol (99.5%) or formalin (10%). Nuclei were stained by the Giemsa procedure. Controls were treated with either deoxyribonuclease or ribonuclease. Ribonucleic acid was stained with acridine orange. Lipid vacuoles were stained with Sudan black B. Glycogen was stained by the periodic acid-Schiff reaction. Metachromatic granules were stained with Bismarck Brown, and cytoplasmic granules were stained with either toluidine blue or azure A. Unfixed preparations were stained for respiratory particles with neotetrazolium hydrochloride (0.1%) in phosphate buffer (pH 6.8, 0.08 M) with either D-glucose (0.01 M) or acetate (0.01 M) as substrate. Because appreciable dye reduction occurred in the absence of exogenous substrate, the basidiospores were placed in a shallow layer of sterile phosphate buffer (0.08 M, pH 6.8), and were allowed to stand overnight; this procedure reduced the endogenous reaction considerably.

Respiration studies. Basidiospores shed from sporulating fruits of *S. commune* accumulate on the petri dish cover and provide a spore print of the gilled fruits (Fig. 1). Spores were collected from 50 petri dish covers of this sort at 18-hr intervals by resuspending the spores in phosphate buffer (pH 6.8, 0.08 M) and centrifugation (2,000 × *g*, 15 min). The basidiospores were finally suspended in 10 ml of phosphate buffer and used directly, or they were frozen and stored at −20 C. To examine the respiratory characteristics of a population of predominantly freshly shed basidiospores, material was collected from 300 cultures of sporulating fruits after a 2-hr deposit and treated as above, with the exception that all subsequent manipulations of harvest were performed at 4 C.

Respiratory rates of basidiospores were measured at 10-min intervals for 1 hr at 30 C by standard manometric techniques. KOH (20%) was in the center well of the vessel; in addition to trapping CO_2 during respiration measurements, this served to arrest basidiospore germination (Hafiz and Niederpruem, 1963). The cyanide mixtures employed for the inhibitor studies were those recommended by Robbie (1958), which involved KCN-KOH mixtures in the center well and KCN in the reaction medium. Dry weights were determined by placing samples in tared weighing cups at 100 C for 24 hr.

RESULTS

Cytochemical studies of basidiospores. In agreement with the early studies of Ehrlich and McDonough (1949), microscopic examination of Giemsa-stained preparations of fresh basidiospores indicated two nuclei in each spore. This reaction decreased markedly with deoxyribonuclease, but the binucleate condition was still evident after ribonuclease treatment. Abundant granules were evident in fresh spores after treatment with certain dyes, including toluidine blue, azure A, and Bismarck Brown. A marked metachromasy was evident with the latter. Carbohydrate appeared to be present in the fresh basidiospore after treatment by the periodic acid-Schiff procedure, whereas no lipid vacuoles were evident with Sudan black B. Acridine orange-stained preparations were light orange when viewed with a fluorescent microscope; this color disappeared after ribonuclease treatment and a faint green color remained. Neotetrazolium-positive particles were observed in the spores after incubation in either D-glucose or acetate.

Basal respiration of basidiospores. The endogenous respiration of basidiospores varied in Q_{O_2} (μliters of O_2 per hr per mg of dry weight) from 10 to 20. Storing the basidiospores for 3 weeks at −20 C did not significantly decrease the magnitude of the basal respiration. Some idea as to the nature of the reserve material supporting the endogenous respiration of fresh basidiospores came from respiratory quotient (RQ) measurements. In this instance, RQ (CO_2/O_2) values near unity were observed. This suggests that carbohydrate may be involved. The finding that fresh basidiospores gave a strong glycogen reaction with appropriate cytochemical procedures lends further support to this notion.

Studies dealing with the effects of the uncoupling agent 2,4-dinitrophenol (DNP) indicated that the endogenous respiratory capacity of basidiospores was considerably greater than the data reported above. Oxygen consumption amounting to 400% of the control respiration was evoked by DNP, and this effect was clearly dependent upon the pH of the reaction medium (Fig. 2). These data point to a very tightly coupled respiratory activity in fresh basidiospores. In addition, the fact that DNP (10^{-5} M) promoted the basal respiration to an even greater degree at pH 3.3 than did DNP (10^{-3} M) at pH 6.8 serves to emphasize the importance of perme-
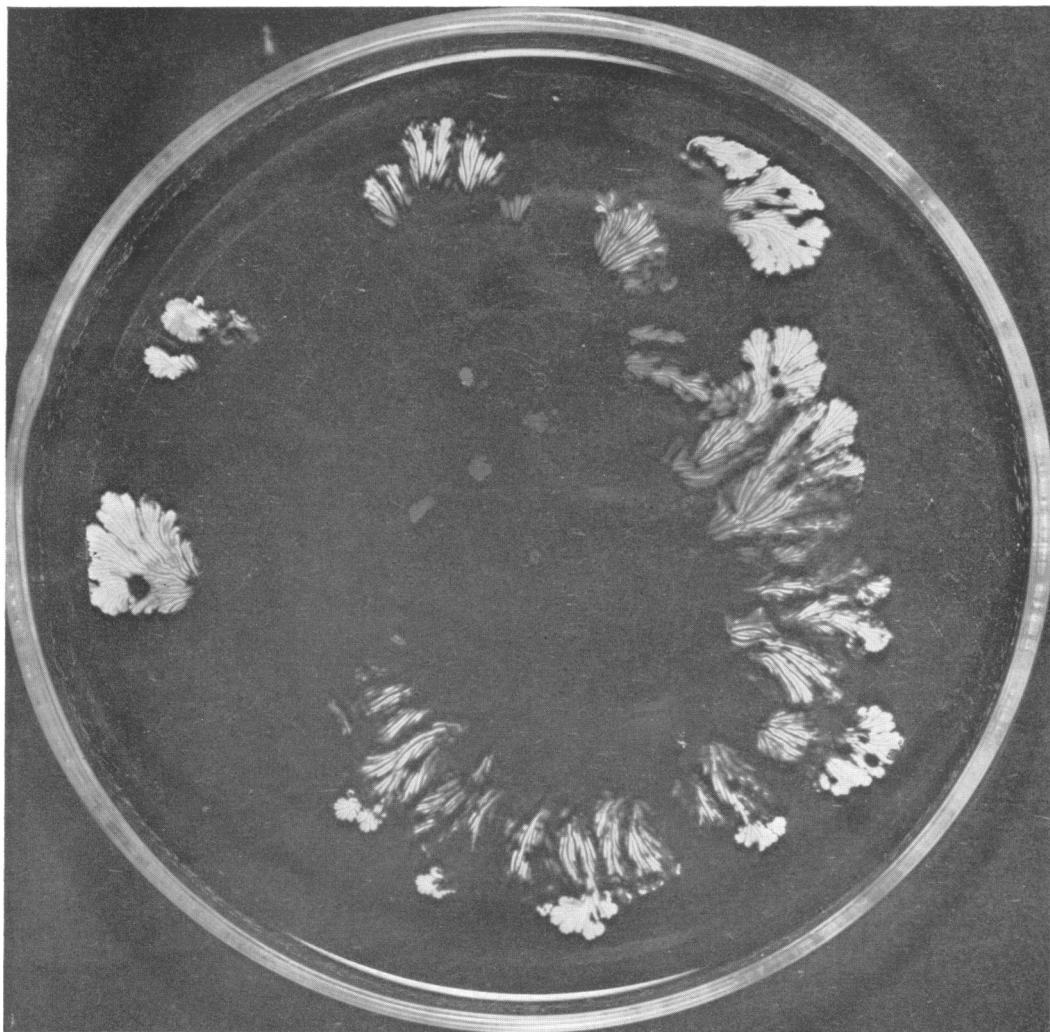
FIG. 1. *Basidiospore print of fruits of Schizophyllum commune.*

ability factors here. A similar situation was described for DNP with yeast (Simon and Beevers, 1952). The DNP-stimulated respiration of basidiospores of *S. commune* was very sensitive to antimycin A, and suggests that probably all of the additional respiration evoked by the uncoupling agent proceeds via a respiratory system involving *b*- and *c*-type cytochrome components.

Substrate utilization by basidiospores. Vegetative mycelium of *S. commune* exhibits an endogenous respiration which is not enhanced significantly by exogenous substrate (Niederpruem

and Hackett, 1961). In contrast, the basidiospores showed characteristic responses to certain exogenous substrates. Respiratory rates amounting to 300 to 400% of the basal respiration were observed with D-glucose. This permitted an evaluation of the role of various carbon sources in basidiospore respiration at pH 6.8 (Fig. 3). Respiratory rates were highest with D-sucrose, D-glucose, D-fructose, D-mannose, L-sorbose, D-xylose, acetate, and ethanol (not shown). No significant increase in respiration was observed with deoxyglucose, certain polyols and amino acids, succinate, pyruvate, glyoxylate, glycolate,
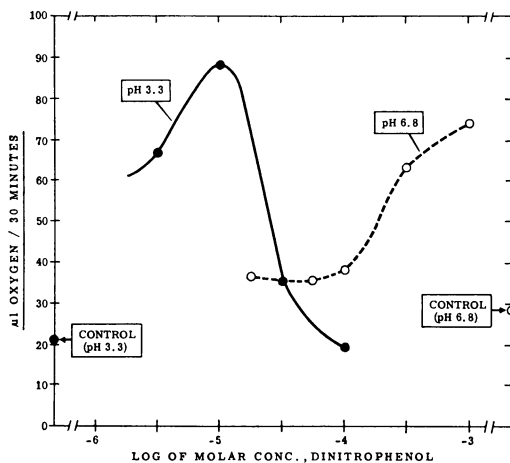
FIG. 2. *Effect of 2,4-dinitrophenol on endogenous respiration of basidiospores of Schizophyllum commune.*

gluconate, D-glucosamine, or D-galactosamine; nor did enhancement occur with succinate, pyruvate, or fumarate when studied at pH 3.0.

The oxidation of D-glucose and acetate did not proceed to completion, and gave only 30 to 50% of the theoretical oxygen consumption required for complete oxidation of these substrates. Addition of fresh substrate again restored the initial high rates obtained with these materials. These data suggest that fresh basidiospores assimilate a considerable fraction of these substrates. A similar situation was described in mycelial pellets of the basidiomycete *Polyporus palustris* (Newcomb and Jennison, 1962).

Although some members of the tricarboxylic acid cycle failed to promote basidiospore respiration, certain enzyme activities, including succinic-cytochrome *c* reductase, isocitric dehydrogenase, and fumarase were demonstrated in cell-free extracts of this material (Ratts et al., 1964). These findings may also relate to permeability barriers associated with the basidiospore.

Inhibitor properties of basidiospore respiration. The respiratory system of vegetative mycelium of *S. commune* (699 $A^{41}B^{41}$) involves the participation of pyridine nucleotide, flavoprotein, and a complete cytochrome system (Niederpruem and Hackett, 1961). The nature of the respiratory system of basidiospores was examined with specific inhibitors (Fig. 4). Oxygen consumption with D-glucose as substrate was reduced significantly in the presence of cyanide (5×10^{-4} M),

azide (10^{-3} M), antimycin A (1 $\mu g/ml$), Atabrine (10^{-3} M), and phenylmercuric acetate (10^{-4} M). The effects of various levels of antimycin A on glucose oxidation are shown in Fig. 5. Alcohol controls were negative in all cases. Inhibition by cyanide and azide implicates cytochrome oxidase, whereas the results obtained with antimycin A are indicative of electron transfer between cytochrome *b* and cytochrome *c*. The sensitivity
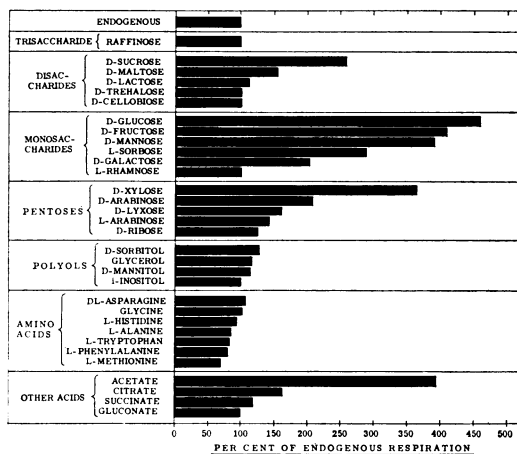

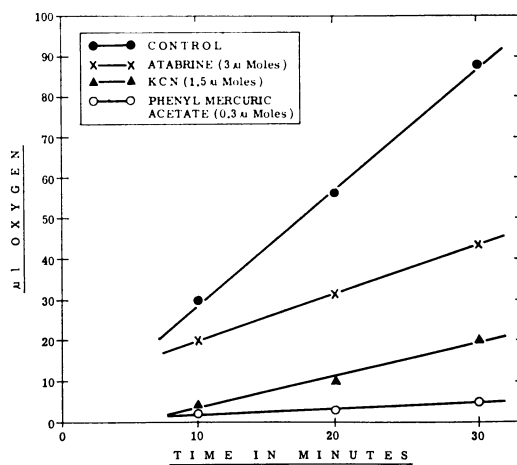FIG. 3. *Patterns of substrate utilization by basidiospores of Schizophyllum commune.*

FIG. 4. *Effects of inhibitors on D-glucose oxidation by basidiospores of Schizophyllum commune. Each respirometer vessel contained (in 2.4 ml): D-glucose, 20 μmoles; phosphate buffer (pH 6.8), 120 μmoles; 0.4 ml of basidiospores (3.3 mg, dry weight); inhibitor as indicated; and 20% KOH (0.2 ml in center well). Temperature, 30 C.*
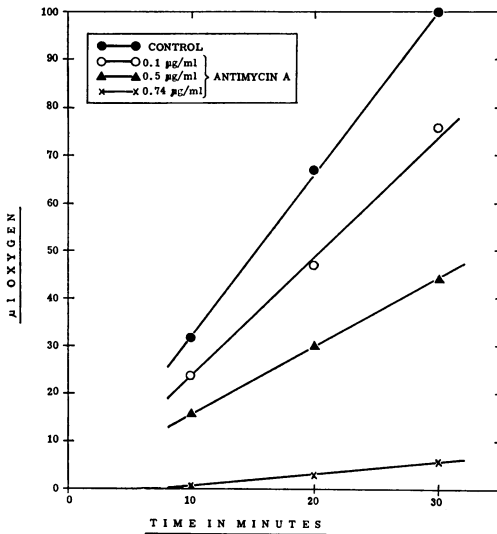
FIG. 5. *Effect of antimycin A on D-glucose oxidation of basidiospores of Schizophyllum commune. Conditions: same as in Fig. 4; antimycin A as indicated.*

to Atabrine suggests a role for flavoprotein, whereas the effects obtained with the organic mercurial point to a requirement for vital sulfhydryl groups in the normal respiration. These findings do not indicate any major qualitative differences between the respiratory chain of the vegetative mycelium and the basidiospores.

No inhibition of glucose respiration was evident with millimolar levels of phenylthiourea, diethyldithiocarbamate, fluoride, amytal, or fluoroacetate. In addition, malonate (0.01 M) failed to inhibit oxygen consumption when applied at pH 2.5. However, experiments with cell-free extracts of this material revealed a malonate-sensitive succinic-cytochrome c reductase (Ratts et al., 1964). This again may reflect permeability barriers in the intact basidiospore to some of these inhibitors.

Amino acid uptake and incorporation by basidiospores. Although amino acids failed to stimulate basidiospore respiration, uptake of certain isotopic L-amino acids was readily demonstrated. Incorporation of L-alanine-1-C^{14} remained linear over a 30-min period at 30 C, and this process was depressed markedly at 4 C. The incorporation of L-alanine-1-C^{14} into trichloroacetic acid-precipitable material was unaffected by chloramphenicol (100 μg/ml), but was inhibited significantly by cycloheximide (25 μg/ml;

Hafiz and Niederpruem, *unpublished data*). It is interesting to note here that mycelial growth of *S. commune* strain 699 is also sensitive to cycloheximide (Parag, 1961).

Effect of basidiospore age. All of the data reported above refer to basidiospores that had accumulated on petri dish covers for 18 hr at room temperature. The possibility existed, therefore, that very young, freshly shed basidiospores may differ appreciably from this material. Accordingly, a 2-hr deposit collected from 300 sporulating cultures was examined for several characteristics associated with 18-hr material. In this regard, significant enhancement of the basal respiration was again observed with D-glucose, acetate, and D-xylose, whereas L-alanine, L-asparagine, and D-ribose were without effect. Inhibitor studies were also performed on the glucose oxidation system. In agreement with data obtained with 18-hr spores, oxygen consumption was depressed markedly by cyanide, Atabrine, and phenylmercuric acetate. These data suggest that no major qualitative differences occurred between spores shed over 2 to 18 hr.

DISCUSSION

This investigation demonstrates that basidiospores of *S. commune* are capable of a variety of aerobic reactions. The DNP-stimulated basal respiration apparently proceeds through a respiratory chain involving *b*- and *c*-type cytochromes, and is indicative of a limitation of phosphate acceptors in vivo. In addition, endogenous respiration may be sustained by carbohydrate rather than lipid. Respiration of dormant ascospores of *N. tetrasperma* involves lipid reserves, whereas immediately after heat activation a carbohydrate fraction involving mainly trehalose supports the 20- to 40-fold increase in basal respiration (Sussman, 1961). Uredospores of the basidiomycete *Puccinia graminis*, on the other hand, utilize lipid as an endogenous fuel for respiration (Shu, Tanner, and Ledingham, 1954).

It is unlikely that basidiospores of *S. commune* are deficient in either enzymes of carbohydrate utilization or energy production. Significant increases in basal respiration are evoked with sucrose, certain hexoses, acetate, ethanol, and D-xylose. The latter also supports growth and indigotin production by mycelium of *S. commune* (Swack and Miles, 1960). Hexokinase and

glucose-6-phosphate dehydrogenase activities were also demonstrated in basidiospore extracts (Hafiz and Niederpruem, *unpublished data*). Moreover, results obtained in the present study with specific metabolic poisons suggest the participation of flavoprotein, *b*- and *c*-type cytochromes, and cytochrome oxidase in basidiospore respiration. Further support for this contention is the finding that reduced nicotinamide adenine dinucleotide oxidase and cytochrome *c* oxidase activities are present in basidiospore extracts; the former is sensitive to Atabrine and antimycin A, whereas both enzyme activities are inhibited by cyanide and azide (Ratts et al., 1964). By these criteria, the respiratory chain of basidiospores differs in no major qualitative way from vegetative mycelium of this mushroom.

A possible clue to the regulatory factors which govern basidiospore germination in *S. commune* may come from studies involving the control of terminal respiration. Spore germination in fungi is often accompanied by increased respiration, and this is also true for *S. commune*. Biochemical studies dealing with basidiospore germination are now in progress.

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