CYTOCHROME SYSTEM IN SCHIZOPHYLLUM COMMUNE[1,2]

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INTRODUCTION

The wood-rotting basidiomycete *Schizophyllum commune* has been of special interest to mycologists since Kniep first outlined the complex pattern of tetrapolar sexuality in its reproductive cycle. In subsequent studies, a great deal of information has been accumulated on the genetics of this organism (16), but relatively little is known about its intermediary metabolism. Wessels (24) has recently demonstrated the operation of the tricarboxylic acid cycle in the breakdown of carbohydrate by *S. commune*. The present report deals with the respiratory system in *S. commune*, with special reference to the mechanisms by which electrons are transferred to oxygen. Although this aspect of metabolism has been studied in great detail with yeast, very little has been done with the basidiomycetes. Experiments carried out with both the intact vegetative mycelium and with cell-free extracts indicate that respiration is mediated by a classical cytochrome-cytochrome oxidase system.

MATERIALS & METHODS

Schizophyllum commune, Fr., strain no. 699, haploid mating type $A^{41}B^{41}$, was grown at 27° C on a solid, minimal medium containing (per liter of distilled water): glucose, 20 g; $(NH_4)_2HPO_4$, 1.5 g; thiamine hydrochloride, 120 μg; KH_2PO_4, 0.46 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; Difco agar, 20 g. In order to follow the growth rate under these conditions, a circular disc (3.5 mm diam) of mycelium was placed as an inoculum on agar in the center of a petri dish, which was then incubated at 27° C and 60 % relative humidity; the diameter of the colony was used as the index of total growth (4). Cultures of mycelial pellets were also grown in aerated liquid media of the same composition, and in this case a macerated mycelial mat was used as the inoculum.

Respiratory rates of the intact mycelium on agar were determined in 3 ml volumetric respirometers by a method developed for slices of plant tissues (21). Mycelium-agar discs (13 mm diam) were removed from the centers of appropriate colonies and the oxygen consumption measured for 1 hour at 25° C. The effects of inhibitors were studied by immersing the discs in buffered inhibitor solutions for 30 minutes and then measuring the respiration. Respiratory rates of the mycelial pellets were followed at 30° C using standard manometric techniques (22). The 72-hour cultures were harvested by centrifugation and the pellets washed with 0.05 M phosphate buffer (pH 7).

To prepare cell-free extracts, the washed mycelial pellets were hand ground with sand in a medium containing 0.05 M phosphate buffer (pH 7) and 0.5 M sucrose. All manipulations were carried out at approximately 4° C. The homogenate was centrifuged at 2,000 × *g* for 10 minutes to remove the abrasive and unbroken filaments. The remaining supernatant was centrifuged at 10,000 × *g* for 20 minutes, and the residue washed by centrifugation and finally suspended in a small volume of isolating medium; this suspension of particles is referred to as the washed mitochondrial fraction, M_w. In an alternative procedure for preparing cell-free extracts, pellets suspended in 0.1 M phosphate buffer (pH 7) were exposed to sonic oscillation (Raytheon, 10 kc) for 15 minutes. After preliminary centrifuging at 2,000 × *g* (10 min) and 10,000 × *g* (20 min), the desired particles were sedimented at 78,410 × *g* for 90 minutes in a preparative ultracentrifuge and finally suspended in the phosphate buffer.

Enzyme activities were followed spectrophotometrically: cytochrome c oxidase and cytochrome c reductase were measured at 550 mμ, and the oxidation of reduced pyridine nucleotides (DPNH, TPNH) at 340 mμ. Organic acid oxidations were measured by standard manometric techniques at 30° C. All reaction vessels included the following cofactors (μmoles): $MgSO_4$, 0.5; cytochrome c, 0.01; diphosphothiamine (DPT), 0.2; coenzyme A (CoA), 0.1; adenosine diphosphate (ADP), 3. Spectrophotometric observations on the turbid particle suspensions were made with a Cary recording spectrophotometer (model 14–M).

RESULTS

RESPIRATION OF MYCELIUM ON AGAR: Since most laboratory manipulations of *S. commune* are carried out with mycelium growing on solid agar medium, the respiratory characteristics were first determined under these conditions. Figure 1 shows the rates of oxygen consumption by discs of mycelium

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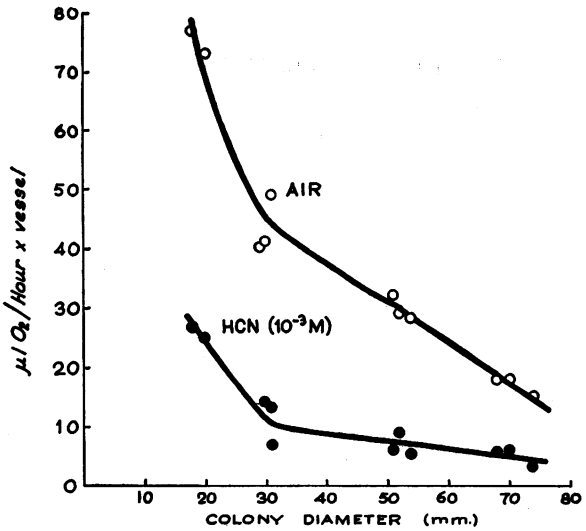


FIG. 1. Effect of culture age on the respiration of discs removed from the center of mycelium on agar. Colony diameters of 20, 30, 50, and 70 mm correspond to 2, 3, 5, and 7 days of growth.

which had been cut out from the centers of colonies of various sizes. The youngest colonies, which had grown for 2 days and reached a diameter of 20 mm, showed the highest respiratory rates (80 μl O_2/hour/vessel containing 2 discs); this rate decreased roughly fivefold after 7 days. A similar rapid decline in respiratory rate takes place during growth of the filamentous fungus *Myrothecium verrucaria* (8). The data of figure 1 also show that the oxygen uptake is markedly inhibited by 10^{-3} M cyanide at all stages of growth examined.

The effects of other respiratory inhibitors were studied using mycelium obtained from colonies of 30 to 40 mm diameter. Sodium azide (10^{-3} M) inhibited the respiration 85 %. Oxygen uptake in the dark was inhibited 45 % by a 95 % CO-5 % O_2 gas mixture, relative to the appropriate N_2/O_2 control. The relative affinity of the oxidase for O_2 and CO, K, calculated according to Warburg's formula (23), varied from 21 to 23. The fact that the CO-inhibition is essentially completely reversed by light (fig 2) strongly suggests that the oxygen-activating enzyme is cytochrome oxidase. The mycelium showed a typical response to the uncoupling agent 2,4-dinitrophenol (DNP): respiration was stimulated 54 % by 10^{-4} M and inhibited 50 % by 10^{-3} M DNP. In parallel studies on the growth of a number of *S. commune* strains, it was found that 10^{-4} M DNP inhibits radial growth approximately 85 %. These results indicate that in *S. commune,* as in other aerobic organisms, interference with oxidative phosphorylation disrupts both the respiratory control and the energy supply for growth.

RESPIRATION OF MYCELIAL PELLETS: The respiratory characteristics of the mycelium were also studied using pellets harvested from liquid cultures after 3 days of aerobic growth. These pellets consumed oxygen at a constant rate (5–8 μl O_2/hr/mg dry wt) for more than 90 minutes. The rates were directly proportional to the amount of mycelium added to the Warburg vessels and they were not enhanced by displacing the air in the gas phase with oxygen.

TABLE I

EFFECTS OF INHIBITORS ON RESPIRATION OF MYCELIAL PELLETS OF SCHIZOPHYLLUM COMMUNE*

INHIBITOR	CONC (FINAL)	% INHIBITION
Cyanide	0.001 M	85
Azide	0.001 M	85
Carbon monoxide (dark)	$CO/o_2 = 19/_1$	50
DIECA	0.001 M	5
PTU	0.001 M	6
Antimycin A	1 μg/ml	95
Phenylmercuric acetate	0.0001 M	97

* The cyanide mixtures were those recommended by Robbie (17). For the CO experiments, control vessels were flushed with a N_2/o_2 ($19/_1$) gas mixture. All the inhibitors were studied at pH 7 with the exception of azide, which was studied at pH 5.

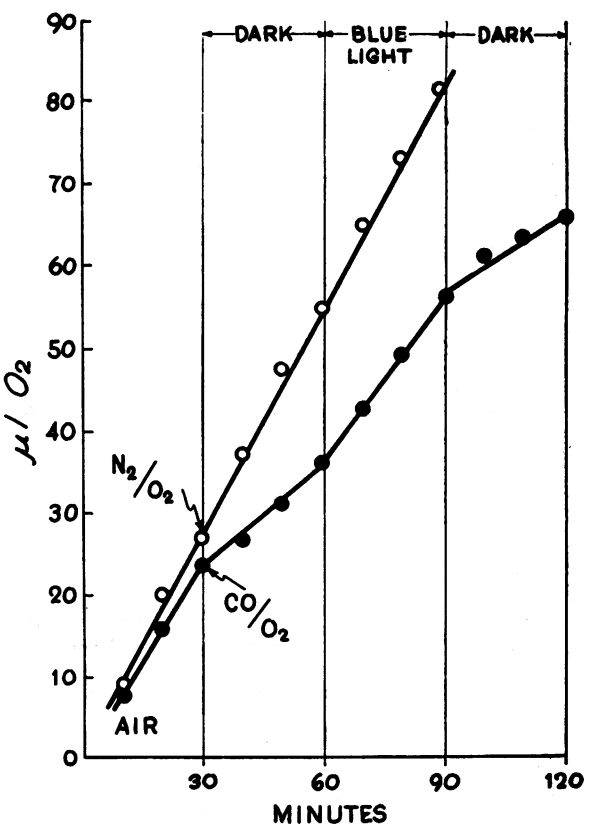


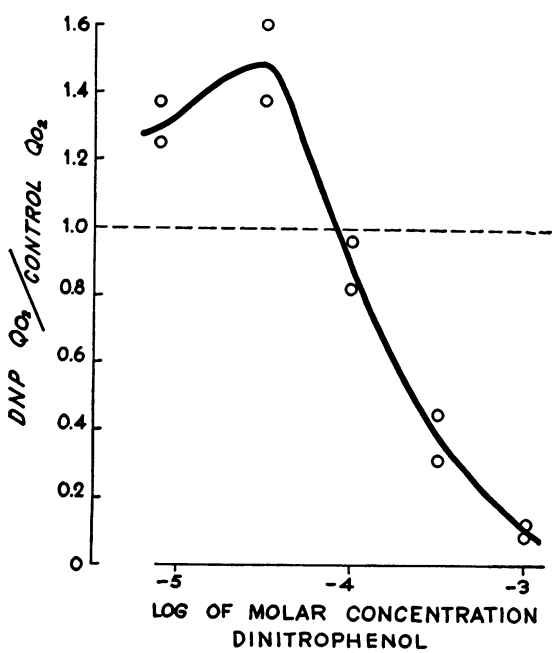
FIG. 2. Effect of carbon monoxide on the respiration of mycelium on agar.

No attempt was made to reduce this endogenous respiration to a point where stimulations by glucose could be demonstrated.

The nature of the respiratory system was examined with the aid of specific inhibitors, and the results are summarized in table I. Oxygen uptake was reduced 85 % by either 10^{-3} M cyanide or azide. In close agreement with the results obtained with the mycelium grown on agar, a 95 % CO-5% O_2 mixture inhibited pellet respiration 50 % in the dark; the 95 % N_2-5 % O_2 control was inhibited 40 %, relative to the rate in air. Millimolar concentrations of two copper chelating agents, diethyldithiocarbamate (DIECA) and phenylthiourea (PTU), inhibited respiration only slightly. Since it is known that these reagents are powerful inhibitors of polyphenol oxidase (12), it would appear that this enzyme, which is widely distributed in the basidiomycetes, does not play a significant role in normal respiration. The respiration was very sensitive to antimycin A, which blocks electron transfer between cytochrome b and c (6), as 1 μg/ml antimycin A (applied in 95 % ethanol) reduced the oxygen uptake to 5 % of the control (containing an equivalent amount of alcohol, which was not itself inhibitory). Phenylmercuric acetate (10^{-4} M), which combines with sulphydryl groups, also inhibited the respiration almost completely. The effects of a wide range of DNP concentrations are shown in figure 3: at low concentrations the oxygen uptake was stimulated as much as 50 %, while high concentrations were inhibitory.

ACTIVITIES OF ISOLATED PARTICLES: Since it is generally agreed that the respiratory chain in aerobic organisms, including the fungi (7), is localized in mitochondria, special attention was focused on the particulate fraction (M_W) isolated from homogenates of hand-ground mycelium. When particles were added to a reaction mixture containing reduced cytochrome c, the optical density at 550 mμ decreased rapidly due to the oxidation of this component (fig 4). (Preliminary studies showed that this cytochrome c oxidase activity decreased 40 % when particles were stored in the frozen state for 1 day, and all subsequent work was carried out with freshly prepared particles.) The rate of oxidation was proportional to the volume of M_W employed and there was no reaction when boiled particles were used. The enzymatic activity was inhibited 80 % by 10^{-3} M azide and 70 % by 10^{-5} M KCN; when the cuvette was flushed in the dark with a 95 % CO-5% O_2 mixture the rate of oxidation was reduced to 50 % of the control rate (N_2/O_2 = 19). All these results indicate that there is a typical cytochrome c oxidase on the particles.

The mitochondrial fraction was also able to oxidize DPNH (fig 5). The rate of DPNH oxidation, which was very slow in the air-saturated buffer medium (0.020 μmoles/min × mg protein), was greatly stimulated by adding 2 × 10^{-5} M cytochrome c (0.179 μmoles/min × mg protein). With cyanide included in the reaction mixture, it was possible to demonstrate



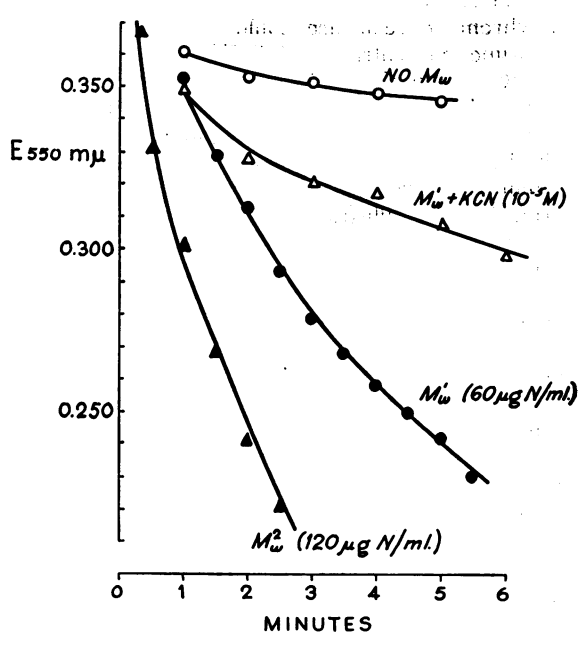


FIG. 3 (*left*). Effects of 2,4-dinitrophenol on the respiration of pellets of mycelium.

FIG. 4 (*right*). Cytochrome c oxidase activity of isolated particles (M_W). Reaction mixture (μM): reduced cytochrome c, 0.01; phosphate, 75; KCN (where indicated), 0.03; particles (6 or 12 μg nitrogen), and water to 3 ml pH 7.0. Room temperature.

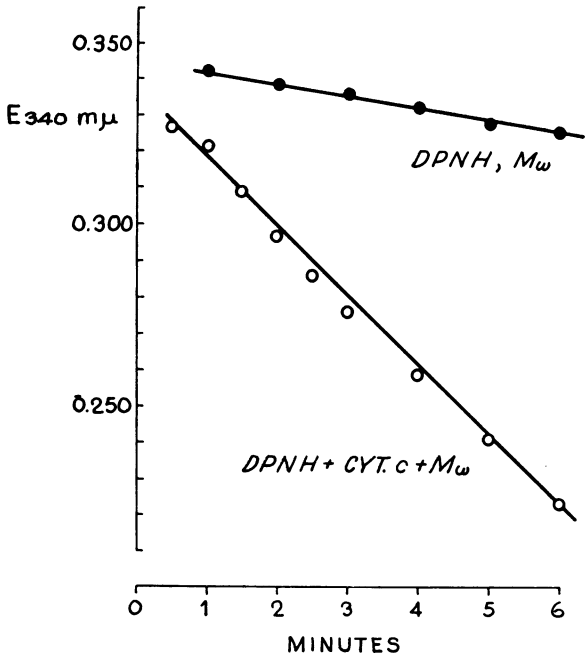


FIG. 5. DPNH oxidase of isolated particles (M_W). Reaction mixture (μM): DPNH, 0.170; cytochrome c (where indicated), 0.01; phosphate, 75; particles (11 μg nitrogen), and water to 3 ml pH 7.0. Room temperature.

the rapid reduction of cytochrome c (fig 6). There was no endogenous reaction and an active DPNH-cytochrome c reductase could be demonstrated; at the same concentration, TPNH was only a tenth as effective an electron donor.

In spite of repeated attempts, it was not possible to detect manometrically any significant oxygen uptake by the particles isolated from hand-ground mycelium using the various tricarboxylic acid cycle intermediates as substrates. However, the particulate fraction obtained after sonic oscillation of the mycelium did consume oxygen at a measurable rate in the presence of succinate. There was no appreciable endogenous respiration, and when succinate was added to the reaction mixture oxygen was taken up at a constant rate (15 μl O_2/hr × mg protein) for at least 20 minutes. Although a wide variety of conditions was tested, it was not possible to demonstrate any esterification of inorganic phosphate during the oxidation of succinate. Under conditions where succinate was oxidized, there was no discernable oxidation of α-ketoglutarate.

SPECTROPHOTOMETRIC OBSERVATIONS ON ISOLATED PARTICLES: The respiratory chain components in particles isolated after sonic oscillation were studied by spectrophotometric techniques. Figure 7 shows a difference spectrum (reduced-oxidized) which compares a suspension of untreated particles with an identical suspension which has been reduced enzymatically by adding DPNH in sufficient excess to exhaust the supply of dissolved oxygen in the cuvette. It is clear that cytochromes of type a (603–605 mμ), b (560–562 mμ), and c (550–552 mμ) are present. The broad band in the 520-530 mμ region represents the fused β-bands of cytochromes b and c. In the Soret region, both the γ-band of cytochrome a-a_3 (443–445 mμ) and the major band of cytochrome b (430 mμ) can be seen; the trough in the 460-480 mμ region indicates the presence of flavoprotein. A rough estimate of the concentrations of the various components can be gained from the difference spectrum, using values for the absorption maxima, reference wave lengths, and extinction coefficients given by Chance and Williams (6). The relative concentrations of the components in the enzymatically-reduced respiratory chain are 1 : 1.4 : 1.6 : 5 for cytochromes a, b, c, and flavoprotein, respectively. It appears that the cytochromes are present in roughly equimolar amounts, whereas flavoprotein is present in molar excess. When the particulate fraction is reduced chemically with sodium hydrosulfite, additional components are reduced (fig 8) and the 445 mμ peak now appears only as a shoulder on the major Soret band.

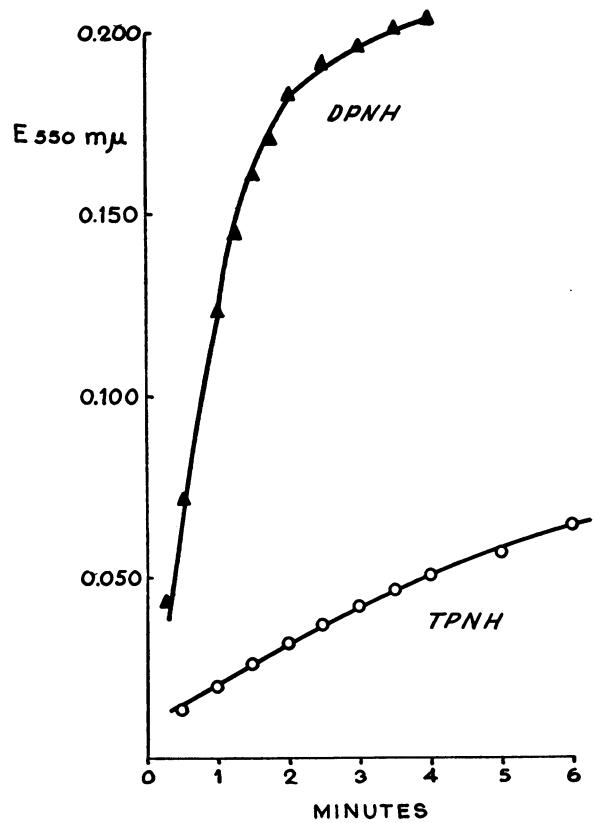
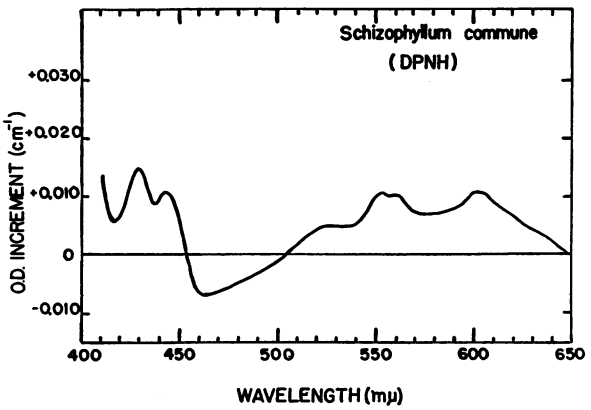


FIG. 6. DPNH- and TPNH-cytochrome c reductase of isolated particles (M_W). Reaction mixture (μM): DPNH or TPNH, 0.120; phosphate, 75; cytochrome c, 0.01; KCN, 0.3; particles (11 μg nitrogen), and water to 3 ml pH 7.0. Room temperature.



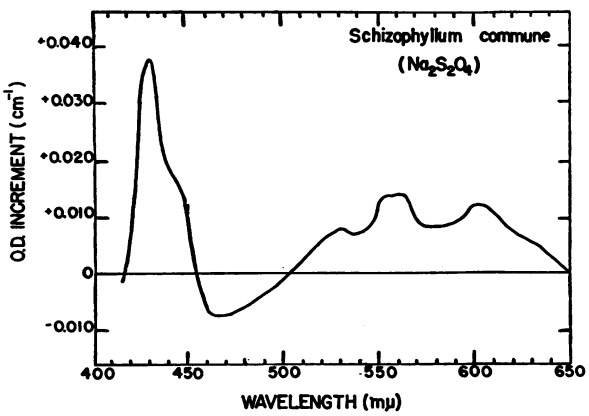


FIG. 7 (*top*). Difference spectrum (reduced with DPNH—oxidized) of isolated particles. Both cuvettes contained 70 μmoles phosphate buffer (pH 7.0), 0.2 ml particles (3 mg protein), and water to 1.5 ml; 4.8 μmoles DPNH added to one. Room temperature.

FIG. 8 (*bottom*). Difference spectrum (reduced with $Na_2S_2O_4$—oxidized) of isolated particles. Both cuvettes contained 70 μmoles phosphate buffer (pH 7.0), 0.2 ml particles (3 mg protein), and water to 1.5 ml; $Na_2S_2O_4$ added to one. Room temperature.

In order to characterize the respiratory pigment which combines with carbon monoxide, the spectral changes caused by flushing an anaerobic suspension of particles with CO were recorded as the (anaerobic + CO)-(anaerobic) difference spectrum. There is a trough at 445 mμ, presumably due to the disappearance of the Soret band of cytochrome a_3, and a broad absorption band between 420 and 430 mμ. The turbidity of the suspensions prevented a more precise definition of the component.

DISCUSSION

The present study has shown that particles isolated from *Schizophyllum commune* contain a typical respiratory chain which can transfer electrons from pyridine nucleotide to molecular oxygen via flavoprotein and the cytochrome system. The effects of respiratory inhibitors on the mycelium lend strong support to the view that this chain is functional in the intact tissue. By blocking electron transfer between cytochromes b and c with antimycin it is possible to eliminate virtually all of the respiration. The nature of the terminal oxidase is well established by demonstrations that A: Mycelium respiration is light-reversibly inhibited by CO and B: The isolated particles show cytochrome c oxidase activity and contain a component which is spectrally similar to cytochrome a_3. Although the rate of respiration declines markedly as the mycelium ages, there is no evidence for any qualitative change in the electron transfer system.

The type of respiratory chain described here is apparently very general among the fungi. Fungal respiration is characteristically cyanide-sensitive (1, 14, 18, 23, 26), although some interesting cases of cyanide- and CO-insensitivity have been reported (9). The respiration of the basidiomycetes *Polystictus versicolor* (2) and *Puccinia graminis* tritici (25) is inhibited by CO and this effect is reversed by light, indicating that here too the electron transfer is mediated by cytochrome oxidase. A spectroscopic survey of 45 species of fungi, including 13 basidiomycetes, showed that a complete cytochrome system was present in every case (3). Cytochrome oxidase activity has been reported in extracts of a variety of fungi (5, 11, 13, 19, 20), and cytochrome c has been purified from the basidiomycete, *Ustilago sphaerogena* (15). The electron transfer system is presumably localized on the mitochondria. No attempt was made in the present study to define the morphology of the *S. commune* particles, but a typical mitochondrial fine structure has been shown in a related basidiomycete, *Coprinus disseminatus* (10).

There is some evidence for the general role of the tricarboxylic acid cycle in the respiration of the fungi (7). Although the *S. commune* particles did oxidize succinate, it was not possible to demonstrate the activity of the entire cycle. After completing this work, the results of Wessels (24), demonstrating the operation of a complete tricarboxylic acid cycle in *S. commune* particles, came to our attention. His isolation procedure differed from those used here in the inclusion of 0.8 M glucose and nicotinamide in the medium; these conditions probably maintain the mitochondria in a more physiological state. Wessels was also able to demonstrate the oxidation of exogenous organic acids by the dried mycelium. The relatively high endogenous respiration of the intact mycelium, and its response to exogenous glucose, may be related to the fact that 10-day-old tissue was used, in contrast to the 3-day-old mycelium employed here.

SUMMARY

The respiratory characteristics of *Schizophyllum commune* mycelium grown on solid and liquid media

have been examined. The oxygen uptake can be markedly inhibited by cyanide, azide, CO (in the dark only), antimycin A, and phenylmercuric acetate, while copper-combining agents have little effect. At low concentrations, DNP stimulates the respiration and inhibits growth, but at higher concentrations both processes are inhibited.

Particles (mitochondria) isolated by differential centrifugation from homogenates of the mycelium showed the following activities: cytochrome c oxidase; DPNH-, TPNH-, and succinic-cytochrome c reductases; DPNH oxidase; succinoxidase. Spectrophotometric observations of the particle components which are reduced by DPNH revealed the presence of roughly equimolar concentrations of a-, b-, and c-type cytochromes and a molar excess of flavoprotein. It is concluded that respiration in *S. commune* mycelium involves the transfer of electrons from substrates to molecular oxygen through a chain which includes these components.

Acknowledgments

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