RESPIRATORY CHAIN OF STREPTOMYCES

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Received for publication August 22, 1960

There is currently a great deal of interest in the streptomycetes as producers of antibiotics, but relatively little is known about the mechanisms of intracellular energy supply in this group of organisms. They are aerobes, and carbohydrates serve as an energy source. There is good evidence that the pathways of carbohydrate metabolism in the streptomycetes include the glycolysis sequence (Cochrane, 1955), the pentose cycle reactions (Cochrane, Peck, and Harrison, 1953; Ganguly and Roy, 1956; Maitra and Roy, 1959), and the Krebs cycle (Cochrane and Peck, 1953; Nickerson and Ram Mohan, 1953; Ganguly and Roy, 1955). However, the pathway of hydrogen (electron) transfer to molecular oxygen has never been studied in detail. Sato (1940) first reported the presence of cytochromes in the Actinomycetes. On the basis of a spectrophotometric survey of the cytochrome components in 13 Streptomyces species, Heim, Silver, and Birk (1957) concluded that a b-type cytochrome is always present, but in no case were they able to detect cytochrome a and in some species cytochrome c was also apparently missing. They suggested that these organisms may have an atypical respiratory chain that terminates with cytochrome b, and this view was subsequently supported by the partial purification of an autoxidizable b-component from extracts of Streptomyces fradiae (Birk, Silver, and Heim, 1957). These findings are not in accord with the spectroscopic observations of Inoue (1958), who detected a-, b-, and c-type cytochromes in mycelium of Streptomyces griseus. We have verified this observation using cell-free extracts of various streptomycetes (Niederpruem and Hackett, 1959). The present paper reports a more complete characterization of the respiratory chain both in the intact mycelium and in particles isolated from S. griseus and S. fradiae.

MATERIALS AND METHODS

Stock cultures. The following organisms were obtained through the courtesy of Dr. P. A. Ark, Department of Plant Pathology, University of California: S. griseus, Streptomyces rimosus, Streptomyces lavendulae, and Streptomyces coelicolor. A culture (no. 11903) of S. fradiae was obtained from the American Type Culture Collection.

Media. Stock cultures were maintained at 30 C on a peptone-glycerol-potato agar medium (distilled water, 750 ml; potato water, 250 ml; peptone, 2.5 g; glycerol, 4 ml; agar, 20 g) and fresh transfers were made every 2 weeks. A variety of pigments are produced by the mycelium when it is grown on this medium. To grow large amounts of essentially colorless medium, yeast extract (1%)-glucose (1%) or nutrient broth (distilled water, 1,000 ml; nutrient broth powder (Difco), 8 g; glycerol, 4 ml; MgSO4, 50 mg) media were employed.

Conditions of culture. Inocula were prepared from sporulating stock cultures by gently scraping the surface of the culture into 5 ml of sterile distilled water, which was then added to 300 ml of medium in a 1-liter Erlenmeyer flask. Aerobic growth conditions were maintained by shaking the flasks vigorously on a platform shaker at 30 C. When the mycelium was to be grown on a large scale (up to 40 flasks), the pellets from a single 24-hr culture served as the inoculum. Dry weights of samples of washed mycelial pellets were determined after drying overnight at 110 C.

Cell-free extracts. The pellets of mycelium which were to be used for the preparation of cell-free extracts were collected on a Büchner funnel and washed with distilled water. The mat of mycelium was scraped from the filter paper and suspended in 0.1 M phosphate buffer (pH 7) with the aid of a power-driven homogenizer.

1 This investigation was supported by a National Science Foundation Grant (to D. P. H.) and by a National Institutes of Health Predoctoral Fellowship (to D. J. N.).

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All subsequent manipulations were carried out at approximately 4°C. The slurry of mycelium was exposed to the action of a sonic oscillator (10 kc), after which the cell fractions, designated according to the nomenclature of Alexander (1956), were isolated by differential centrifugation. The sonic extract was first centrifuged at 2,000 × g for 15 min; the 2s15 fraction was then centrifuged at 10,000 × g for 15 min. The sediments from both of these centrifugations were made up largely of mycelial filaments. The protein content (Lowry et al., 1951) of the 10s15 fraction was determined as a function of the time of sonic treatment and this value reached a maximum after 5 min; in all subsequent experiments, the sonic treatment was carried out for 5 to 10 min. The 10s15 fraction was centrifuged in a Spinco preparative ultracentrifuge at 78,000 × g for 90 min, and the translucent gold-brown pellet was resuspended in a small volume of 0.1 M phosphate buffer (pH 7) and used as the cell-free extract (78sp90).

Oxidative activities. The respiration of the washed intact mycelium and the succinoxidase activity of the cell-free extracts were measured at 30°C using standard manometric techniques (Umbreit, Burris, and Stauffer, 1957). The succinoxidase activity decreased markedly after storing the particles for 24 hr at 4°C, so that all experiments were carried out with fresh extracts. The oxidation or reduction of mammalian cytochrome c was followed spectrophotometrically at 550 mμ; the oxidation of reduced pyridine nucleotides was followed at 340 mμ. Diaphorase activity was determined by measuring the reduction of 2,6-dichlorophenolindophenol by reduced diphenylpyridine nucleotide (DPNH) at 600 mμ. The molar extinction coefficients for DPNH and 2,6-dichlorophenolindophenol were taken as 6.22 × 10^4 cm^2 per mole (Horecker and Kornberg, 1948) and 16.1 × 10^4 cm^2 per mole (Crane, 1957), respectively. For the inhibitor studies, fresh solutions of sodium cyanide and 5-ethyl-5-isooamylbarbiturate (amytal) were prepared daily.

Difference spectra. Difference (reduced - oxidized) spectra of the cell-free fractions were obtained using a Cary model 14 recording spectrophotometer. Enzymatic reduction of the respiratory chain components was achieved using either DPNH or sodium succinate, which were added in sufficient quantity to exhaust the supply of dissolved O_2 in the cuvette; sodium hydrosulphite was used as a chemical reductant.

RESULTS

Respiration of mycelium. Preliminary studies were carried out to survey the respiratory characteristics of mycelium grown aerobically in nutrient broth. The endogenous respiratory rates (Q_O_2 = μl O_2/hr × mg dry weight) for pellets of S. griseus, S. coelicolor, and S. fradiae were 21, 24, and 13, respectively. The oxygen uptake could be inhibited between 50 and 70% by 10^-3 M cyanide in every case. A cyanide-sensitive respiration was also demonstrated in S. lavendulae and S. rimosus. The effects of several other respiratory chain inhibitors were tested on S. fradiae: Antimycin A and 2-heptyl-4-hydroxyquinoline N-oxide, which block electron transfer in mammalian systems between cytochromes b and c, were only slightly (10 to 15%) inhibitory, and the same was true for amytal, which blocks between DPNH and flavoprotein. A 95% CO-5% O_2 mixture caused no significant

![Fig. 1. Succinoxidase activity of 78sp90 fraction. The reaction mixture contained: sonic extract (14 mg protein); potassium phosphate buffer (pH 7.0), 150 μmoles; where indicated, sodium succinate, 60 μmoles, and sodium cyanide, 3 μmoles; water to 3.0 ml. Cyanide mixtures made up according to Robbie (1948). Temperature, 30°C.](http://jb.asm.org/)
inhibition of the respiration in the dark. Nevertheless, the fact that cyanide did inhibit respiration suggested that a cytochrome system may in fact be functional in the mycelium.

Activities of cell-free extracts. The 78p90 fraction isolated after sonic disruption of *S. fradiae* and *S. griseus* oxidized succinate readily with the consumption of from 5 to 14 μl of O₂ per hr per mg protein (Fig. 1). This succin oxidase activity was very sensitive to cyanide, but CO, Antimycin A, and 2-heptyl-4-hydroxyquinoline N-oxide had relatively little inhibitory effect. The similarity in the pattern of inhibition of mycelium respiration and particle succin oxidase suggests that the same respiratory chain is probably involved in both cases. Oxygen uptake could not be demonstrated when α-ketoglutarate, malate, or citrate, was supplied as the substrate.

Since the dehydrogenases for these substrates were demonstrable in crude extracts when a dye was included as electron acceptor (Cochrane and Peck, 1953; Nickerson and Ram Mohan, 1953), the link to the respiratory chain appears to have been disrupted during the isolation of the particles. No significant succin oxidase activity could be detected in either the 10p15 fraction or in the 78s90 fraction from *S. fradiae*.

The 78p90 fraction was also able to oxidize DPNH at the expense of molecular oxygen, but the rate varied markedly with the age of the culture (see below). The DPNH oxidase of *S. griseus* particles was almost completely blocked by 10⁻⁴ M amytal and it was inhibited 62% by 10⁻⁵ M 2-heptyl-4-hydroxyquinoline N-oxide (Fig. 2). On the other hand, this system was relatively insensitive to cyanide, the degree of inhibition varying from 0 to 50%, depending on the age of the cultures. The oxidation of DPNH could also be coupled to 2,6-dichlorophenolindophenol reduction: there was no endogenous reduction of the dye, but it was rapidly reduced.
Fig. 4. Difference spectra (reduced with Na$_2$S$_2$O$_4$-oxidized) of 78p90 fractions isolated from various *Streptomyces* sp. Protein concentrations (mg/ml): *S. rimosus*, 2.9 (Soret region) or 5.7 (visible region); *S. griseus*, 1.4; *S. fradiae*, 1.5. Room temperature.

It is of interest that the *S. griseus* particles were able to oxidize mammalian cytochrome c at a very slow but measurable rate (Fig. 3). The initial decrease in optical density at 550 m$\mu$ was only 0.0035 per min per mg protein. This reaction was completely blocked by 3 $\times$ 10$^{-4}$ M cyanide, whether applied initially or after roughly a third of the substrate was oxidized (Fig. 3). Comparable cytochrome c oxidase activities were observed with *S. fradiae* and *S. coelicolor* particles. It was not possible to demonstrate any significant DPNH-cytochrome c reductase activity.

The fact that these preparations are relatively...
inactive toward mammalian cytochrome c does not eliminate the possibility that they might be very active with the homologous streptomycyes cytochrome.

**Difference spectra.** The respiratory chain components in the 78p90 fraction were observed by direct spectrophotometric examination of the turbid suspensions. When hydrosulfite is used as the reducing agent, the difference spectra (Fig. 4) show characteristic absorption bands of a-, b-, and c-type cytochromes at 600, 562 to 559, and 552 to 550 μm, respectively; the single Soret band, whose peak varied from 428 to 434 μm, suggests that the b-type cytochrome is predominant. The α-peaks of the three characteristic cytochromes could be identified in all five species examined. Only in the case of S. griseus did the hydrosulfite difference spectrum show a shoulder at 445 μm, corresponding to the γ-band of an a3-type cytochrome. The decrease in absorbancy at 460 to 475 μm suggests the presence of flavoproteins, whose absorption in this region decreases on reduction. In addition to these components in the particles, the final supernatant fractions also contained hydrosulfite-reducible, cytochrome-like materials; their absorption maxima in the Soret region varied between 422 and 426 μm, depending on the species.

To further delineate the α-type cytochrome, a fine stream of CO was bubbled through the hydrosulfite-reduced suspension of S. griseus particles, and the difference spectrum (reduced + CO − reduced) recorded (Fig. 5). There was a marked decrease in absorption at 445 μm, suggesting that the component which combines with CO resembles cytochrome a3; the increase in absorption at 428 μm is presumably due to the CO-complex. The valley in the CO difference spectrum of S. fradiae particles was at 442 μm when the cultures were grown for 18 hr, but this shifted to 430 μm after 37 or 61 hr of growth (Fig. 5).

When succinate or DPNH was used to reduce the respiratory chain enzymatically, some of the components could be seen more clearly than when hydrosulfite was the reductant. The difference spectrum of succinate-reduced S. fradiae particles shows the Soret band of the a-type cytochrome (442 μm), as well as the characteristic α-peaks of cytochromes a, b, and c (see Fig. 2 in Niederpruem and Hackett, 1959). The difference spectrum of DPNH-reduced S. griseus particles shows these same components, and the cytochromes c:b ratio is much higher than in the hydrosulfite-reduced preparations (Fig. 6). The effects of CO on these spectra were qualitatively similar to those observed with the hydrosulfite-reduced suspensions. From these
results it may be concluded that the normal respiratory substrates reduce a complete cytochrome system, including a terminal \( a \)-type component which can react with both \( O_2 \) and CO.  

**Effects of culture age.** A number of observations made in the course of these studies suggested that the age of the Streptomyces cultures was an important factor in determining the nature and activity of the respiratory systems. In the case of *S. fradiae* growing in yeast extract-glucose medium, the endogenous respiration of the mycelium, the DPNH oxidase, and the succinoxidase of isolated particles were all highest during the first 18 hr of growth, after which they declined rapidly. The color of the 78p90 fraction changed from a gold-brown at 18 hr to gray at 37 hr to black at 61 hr. At the later stages, it was increasingly difficult to demonstrate the cytochrome \( a \) bands in the difference spectra. Nevertheless, cyanide remained an effective inhibitor of the mycelium respiration. With *S. griseus*, on the other hand, the mycelium respiration remained high during 50 hr of growth, and the ability of the 78p90 fraction to oxidize DPNH increased 5-fold over this period. As the rate of DPNH oxidation increased, there was a marked increase in the sensitivity of this reaction to cyanide and amytal. The difference spectra of these particles showed an increasing predominance of the \( b \)-type cytochrome.  

**DISCUSSION**

The hypothesis that respiration in the streptomycetes involves a truncated respiratory chain, terminating with a cyanide-insensitive, autoxidizable cytochrome \( b \)-type component (Heim et al., 1957), is not supported by the present study. All of the evidence indicates that a complete cytochrome system is functional in members of this genus. The oxygen uptake by the intact mycelium and the succinoxidase activity of the isolated particulate fraction could always be inhibited by \( 10^{-3} \) M cyanide. The sensitivity of the DPNH oxidase system to amytal implicates flavoproteins in the respiratory chain, and the inhibition by 2-heptyl-4-hydroxyquinoline \( N \)-oxide suggests a flow of electrons between \( b \)- and \( c \)-type cytochromes. Finally, the spectrophotometric evidence indicates that the cytochrome system, including an \( a_3 \)-like component which combines with CO, can be reduced enzymatically by succinate and DPNH. It is reasonable to assume that the normal respiratory electron transfer from substrates to oxygen proceeds successively through pyridine nucleotide, flavoprotein, \( b \)-, \( c \)-, and \( a \)-type cytochromes, and terminates with the cyanide-sensitive cytochrome oxidase. The independent spectroscopic observations of Inoue (1958) on *S. griseus* mycelium are in agreement with this conclusion. As discussed earlier (Niederpruem and Hackett, 1959), the results of Heim et al. (1957) can probably be attributed to the fact that the \( c \)- and \( a \)-type cytochromes are present in relatively low concentrations in whole homogenates, and they can only be shown clearly in the particulate fraction. 

Several experimental observations remain to be explained. The DPNH oxidase system of particles isolated from young mycelium (9 to 18 hr) was somewhat resistant to cyanide. The fact that both the mycelium respiration and the particulate succinoxidase were cyanide-sensitive at this time suggests that the inhibitor-resistant pathway is not physiologically important. The endogenous respiratory rate and the DPNH oxidase activity at this stage were relatively low so that cytochrome oxidase may have been present in sufficient excess, relative to the rate-limiting reaction, to account for the resistance to inhibitors. A similar argument could be used to explain the amytal-insensitivity of particles isolated from young cultures. The nature of the CO-combining pigment(s) in particles isolated from old cultures of *S. fradiae* is not clear. The spectra suggest that this component has a Soret peak at 430 \( \mu \text{m} \), instead of 442 \( \mu \text{m} \), and its CO-complex absorbs at 418 to 420 \( \mu \text{m} \). This apparent shift in the absorption maximum may be due to interference from a denatured \( b \)-type cytochrome, which would also combine with CO. Alternatively, an additional, native CO-binding pigment, such as cytochrome \( o \) (Castor and Chance, 1959), which forms a CO-complex with absorption maximum near 418 \( \mu \text{m} \), may have been present, but the available evidence does not permit a decision on this point.  

**SUMMARY**

Some respiratory characteristics of five *Streptomyces* species (*S. griseus*, *S. rimosus*, *S. lavendulae*, *S. coelicolor*, and *S. fradiae*) have been examined. The endogenous respiration of the mycelium of each species was inhibited from
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50 to 70% by $10^{-3}$ m cyanide. Particles (78p90) isolated by differential centrifugation from sonic extracts showed the following activities: diphosphopyridine nucleotide (DPNH) oxidase, DPNH diaphorase, and succinidase. The DPNH oxidase could be inhibited by amytal, 2-heptyl-4-hydroxyquinoline N-oxide, and cyanide, although the degree of inhibition varied with the age of the cultures. The succinidase was sensitive to cyanide, as was the slow oxidation of mammalian cytochrome c by the particles. Direct spectrophotometric observations of the particle suspensions, reduced enzymatically with DPNH or succinate, have demonstrated the presence of a-, b-, and c-type cytochromes, and probably flavoprotein. Hydrosulfite reduces additional cytochrome b-like material in the particulate fraction. It is concluded that, in the streptomycetes, transfer of electrons from substrates to oxygen involves a complete cytochrome system.

REFERENCES


