Electron Transport in Neurospora Mitochondria

STUDIES ON WILD TYPE AND POKY*

ALAN M. LAMBOWITZ,† EDWIN W. SMITH,§ AND CAROLYN W. SLAYMAN‖

From the Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06510, the Department of Biology, Case Western Reserve University, Cleveland, Ohio, and the Departments of Microbiology and Physiology, Yale University, New Haven, Connecticut 06510

SUMMARY

A comparative study has been made of mitochondria isolated from wild type Neurospora and from poky, a cytochrome-deficient mutant. Both types of mitochondria respire with a variety of Krebs cycle acids and with NADH, but State 3 respiration rates are typically 20 to 40% lower in poky than in wild type. The dehydrogenases and flavoproteins of the two strains appear qualitatively similar, as judged by the dependence of respiration rate on substrate concentration and by the effect of pyrrolnitrin, an inhibitor that acts in the flavin region.

The major difference between wild type and poky is in their oxidase systems. In wild type, more than 90% of the respiration is mediated by a conventional cytochrome chain (sensitive to antimycin and cyanide), whereas poky uses both a cytochrome chain and an alternate oxidase (resistant to antimycin and cyanide but sensitive to salicyl hydroxamic acid). The alternate oxidase accounts for as much as 70% of the respiration in poky mitochondria.

Two lines of evidence suggest that, in poky, the cytochrome chain and the alternate oxidase compete for reducing equivalents on the oxygen side of the dehydrogenases, and thus form a branched electron transport system. (a) Total State 3 respiration rates are significantly lower than would be predicted from the sum of the two oxidase activities measured separately. (b) In addition, it is possible to demonstrate a rapid, energy-dependent reduction of the alternate oxidase by N,N,N',N'-tetramethyl-p-phenylene-diamine dihydrochloride plus ascorbate (via cytochrome c); the deficiency of Site 1 in poky mitochondria (see next paper) makes it unlikely that electrons pass from cytochrome c through the dehydrogenases in this experiment.

Poky (ni-1) is perhaps the best known of the cytochrome-deficient mutants of Neurospora crassa (1), containing abnormally low amounts of cytochromes aa3, b, and c1 but an excess of soluble cytochrome c and flavoproteins (2-5). About one-third of the respiration of poky cells is thought to occur by way of the cytochrome system since it is sensitive, under the appropriate experimental conditions, to low concentrations of antimycin and cyanide (6). The remainder of poky's respiration is mediated by an alternate oxidase which is resistant to antimycin and cyanide (6-8) and does not use cytochrome components (3).

We have recently shown that respiration through the alternate oxidase is specifically blocked by SHAM (Reference 6). The present report, part of our continuing study of cyanide-resistant respiration in Neurospora, summarizes a series of respiration experiments on mitochondria from the poky and wild type strains. Our results suggest that the cytochrome system and the alternate oxidase of poky mitochondria use the same set of dehydrogenases and form a branched electron transport pathway with the branch point located in the flavin region. A characterization of respiratory states and oxidative phosphorylation is presented in the next paper.

MATERIALS AND METHODS

Strains of Neurospora

The poky strain was NSX f+ a and was obtained from Rifkin and Luck (4). f+ is a nuclear gene suppressor of the poky mutation, increasing the growth rate of strains which carry it without restoring the wild type respiratory system (9). The wild type strain was RL21a, derived from the original Lindegren wild types L1A and L25a (Reference 10).

*This work was supported by United States Public Health Service Research Grant GM 15761 from the National Institute of General Medical Sciences. Address reprint requests to Department of Physiology, Yale University, New Haven, Connecticut 06510.
† A. M. L. was a Predoctoral Trainee of the Public Health Service during the course of this work. Present address, the Johnson Foundation, University of Pennsylvania, Philadelphia, Pennsylvania 19104.
§ E. W. S. held a Public Health Service Predoctoral Fellowship during the course of this work.
‖ C. W. S. holds a Research Career Development Award (GM 20185) from the National Institute of General Medical Sciences.

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Growth of Cells

Cells were grown in aerated liquid culture at 25° in Vogel's (11) minimal medium plus 2% sucrose, and were harvested during late exponential phase; 15.5 hours for wild type and 21 hours for poky (6).

Preparation of Mitochondria

Mitochondria were prepared by a modified snail enzyme procedure (12). Cells were harvested by filtration and washed, first with distilled water, and then with protoplast incubation medium (0.59 M sucrose, 0.05 M NaH2PO4, 0.005 M EDTA, pH 6.8). About 8 g wet weight of cells were suspended in 25 ml of this medium containing in addition 10 mM 2-mercaptoethanol (Baker) and 1.4 ml of partially purified snail digestive juice (Glasulase, Endo Products Laboratories, Garden City, New York). The cell suspension was incubated for about 1 hour at 30°. At that time, microscopic examination showed that approximately 70% of the cells would lyse when placed in distilled water. After the snail enzyme treatment, the cells were washed in 200 ml of 0.69 M sucrose and centrifuged at 1000 × g for 5 min. The resulting pellet was mixed with 350 ml of mitochondrial preparation medium (0.33 M sucrose, 1 mM EGTA, 0.3% bovine serum albumin (Fraction V, Sigma) pH 7). The decreased osmolarity of this medium caused the cells to swell and burst through the weakened cell walls, releasing swollen protoplasts and cell contents. This suspension was then homogenized gently at low speed in a Teflon-glass homogenizer to ensure complete breakage of the protoplasts. The homogenate was centrifuged at 1000 × g for 10 min to remove whole cells and nuclei; and the resulting supernatant centrifuged at 9000 × g for 15 min. This high speed spin gave a reddish-brown mitochondrial pellet covered with a tan fluffy layer which could be removed by gently swirling a small amount of supernatant over the pellet. Particularly in the case of poky mitochondria, the quality of the preparation depended to a great extent on the complete removal of the fluffy layer. The remaining pellet was resuspended in 150 ml of mitochondrial preparation medium and put through a second cycle of differential centrifugation, 600 × g for 10 min and 9000 × g for 15 min. The final mitochondrial pellet was resuspended by hard pipetting to give about 0.6 ml of a suspension containing 40 to 60 mg of protein per ml.

Wild type mitochondria isolated by this procedure showed good respiratory control with NADH-linked substrates and contained high concentrations of cytochrome c and pyridine nucleotides (5). These properties are often taken as characteristics of undamaged mitochondrial preparations (13). In contrast, the poky mitochondria used in the present experiments showed poorer respiratory control (see next paper) and contained lower concentrations of pyridine nucleotides than did wild type (5). It is not clear whether these properties reflect damage to the mitochondria during isolation, or are characteristic of poky mitochondria in vivo. For both strains, electron microscopy showed mitochondria which appeared generally (80 to 90%) intact, and which were relatively free of particulate contamination.

Respiration Measurements

Oxygen uptake was measured polarographically with a Clark oxygen electrode (Yellow Springs Instruments, Cleveland, Ohio). Reactions were carried out at 25° in a Lucite chamber containing 2.2 ml of respiration medium (0.3 M sucrose, 8 mM NaH2PO4, 8 mM Tris, 5 mM MgCl2, 0.7 mM EDTA pH 7.2) saturated with air (240 μM O2). 0.2 to 1.5 mg of mitochondrial protein were used per incubation. Unless otherwise indicated, respiration rates reported in the text are maximal State 3 rates (that is, ADP was not rate limiting; reference 14), expressed in μatoms oxygen consumed per min per mg of mitochondrial protein.

Protein Determination

Protein was assayed by the method of Lowry et al. (15) using bovine serum albumin as a standard.

Materials

Substrates—Pyruvate, malate, α-ketoglutarate, succinate, and NADH (all obtained from Sigma), N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (TMPD; Eastman) and ascorbate (Baker) were used as substrates. TMPD was dissolved in double distilled water, and all other substrates were dissolved in respiration medium (see above) and neutralized. The concentration of NADH was calculated from its absorbance at 340 nm using a millimolar extinction coefficient of 6.22; all other substrate concentrations were measured gravimetrically. With the exception of pyruvate, substrates were prepared as concentrated solutions and stored frozen. Dilute solutions of pyruvate (5 mM) were prepared fresh for each experiment in order to minimize dimerization (16).

Adenine Nucleotides—ADP and ATP (both obtained from Sigma) were dissolved in double distilled water and adjusted to pH 6.8. Their concentrations were calculated from the absorbance at 260 nm using a millimolar extinction coefficient of 15.4.

Inhibitors—Rotenone (Sigma), amytal (Lilly), pyruvobinitir (Lilly), antimycin (Sigma), SHAM (Aldrich), oligomycin (Sigma), and carbonyl cyanide m-chlorophenylhydrazone (CCCP; Cal-Biochem) were dissolved in absolute ethanol. Sodium cyanide (Baker) was dissolved in double distilled water and neutralized, and its concentration was checked by titration. Cyanide and rotenone were prepared fresh for each experiment, and all other inhibitors were prepared fresh whenever respiration was measured as a function of inhibitor concentration.

RESULTS

Respiration Rates

Both wild type and poky mitochondria respired with a wide range of substrates, including several Krebs cycle acids and exogenous pyridine nucleotides; State 3 respiration rates with these substrates were typically 20 to 40% lower for poky than for wild type, however (Table I). In both strains, respiration with 5 mM pyruvate required the presence of 1 mM malate. Malate also shortened the time required to attain a maximal State 3 rate with α-ketoglutarate (5 mM), presumably by facilitating substrate penetration (17, 18). Malate itself was oxidized very slowly, even at concentrations as high as 20 mM (Table I).

Stability of Respiration

For further experiments, pyruvate (plus malate), succinate, and NADH were chosen as primary substrates. It seemed important to investigate the stability of respiration rates with these substrates since Eakin and Mitchell (8) reported that suc-
Respiration rates (mean ± S.E.M.) were measured as described under "Materials and Methods." For poky f+ mitochondria, the rates were for preparations less than 1-hour-old. Substrate concentrations were NADH (0.33 to 1.1 mM), succinate (10 to 20 mM for wild type; 20 mM for poky f+), pyruvate (5 mM), a-ketoglutarate (5 mM), malate (1 mM when used in combination with pyruvate or a-ketoglutarate; 20 mM when used separately), TMPD (0.2 mM), ascorbate (10 mM). (TMPD, 0.2 mM, in combination with ascorbate gives a maximal rate for poky f+ but not for wild type.) In all cases, the ADP concentration (0.19 to 0.80 mM) was sufficient to give a maximal State 3 rate.

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Respiration rates in wild type and poky f+ mitochondria with various substrates

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Respiration rates as a function of substrate concentration.

Respiration rates were measured as a function of substrate concentration to gain information, in poky compared with wild type mitochondria, about the early steps in substrate utilization (specific substrate transport systems and dehydrogenases or both). $K_{1/2}$ values and $V_{max}$ values, calculated from Lineweaver-Burk plots, are summarized in Table II. The $K_{1/2}$ values for NADH and pyruvate (plus malate) agreed closely for mitochondria isolated from the two strains, but the $K_{1/2}$ for succinate was somewhat higher in poky than in wild type mitochondria.

Effect of respiratory inhibitors

The remaining portions of the wild type and poky electron transport systems were compared with a series of respiratory inhibitors.

Rotenone and Amytal—Rotenone and amytal are widely used to block electron transport in the NADH dehydrogenase region (19-25); in most mitochondria they inhibit respiration maximally at concentrations of 10 μM and 2 mM, respectively. In both wild type and poky mitochondria, however, rotenone (10 μM) did not inhibit more than 15%, and amytal (2 mM) not more than 25% of the respiration with any substrate tested. At a higher concentration amytal (10 mM) caused somewhat more inhibition (30 to 75%), but the fact that respiration with succinate was affected as much as respiration with pyruvate (plus malate) or NADH indicated that amytal was not acting specifically in the NADH dehydrogenase region.

Pyrrnlnitrin—Pyrrnlnitrin has been reported to inhibit electron transport in the flavin region of animal and yeast mitochondria (26, 27). Recent experiments in this laboratory have confirmed this site of action in Neurospora but have indicated two additional effects; uncoupling of oxidative phosphorylation, and at high concentrations (above 30 μg per ml), inhibition of cytochrome oxidase. Particularly because of the latter effect, it is important to examine the action of pyrrnlnitrin as a function of concentration if one wishes to use this inhibitor to compare the flavin regions of different types of mitochondria. Fig. 1 illustrates the effect of pyrrnlnitrin on the NADH oxidase activity of wild type and poky mitochondria. Half-maximal inhibition occurred at similar concentrations in the two strains; 16 μg per ml in wild type and 12.5 μg per ml in poky. Since these concentrations are below the range in which pyrrnlnitrin affects cytochrome oxidase, the results point to a similarity in the flavin regions of wild type and poky.

Antimycin and Cyanide—In animal mitochondria, antimycin and cyanide are effective inhibitors of electron transport, acting between cytochromes b and c1 and at cytochrome aa₃, respectively (28-30). In wild type Neurospora mitochondria, respiration with all substrates tested was sensitive to low concentrations of cyanide or antimycin (Figs. 2 and 3). Half-maximal inhibition of NADH and succinate oxidase activity occurred at 0.011 and 0.016 mM cyanide, respectively. Inhibitory concentrations of antimycin varied somewhat from one mitochondrial preparation to another (one set of data shown in Fig. 3), but in every preparation the same concentration (between 0.054 and 0.690 μg per mg of protein) gave half-maximal inhibition of both NADH and succinate oxidase activity. The fact that NADH and succinate oxidase have similar sensitivities to cyanide and

Table II

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<td>Pyruvate (plus malate)</td>
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antimycin, and the other (the alternate oxidase) blocked by cyanide system; Reference 5) blocked by 1 innI cyanide or by SHAM in poky mitochondria; one (previously shown to be a cytochrome system) was substituted for cyanide.

Fig. 1 (left). Effect of pyrrolnitrin on NADH oxidase in wild type and poky f+ mitochondria. Control rates (in the presence of 0.75 mM NADH and 0.65 mM ADP) were 1.2 to 1.3 and 0.43 to 0.48 amoloms of O per min per mg of protein for wild type and poky f+ mitochondria, respectively. Pyrrolnitrin was added during State 3, and inhibited rates were measured 1.5 to 2 min after addition. Similar protein concentrations (0.15 and 0.18 mg per ml for wild type and poky f+ mitochondria, respectively) were used for both strains since sensitivity to pyrrolnitrin was found to be somewhat dependent on protein concentration.

Fig. 2 (center). Effect of cyanide on NADH oxidase in wild type mitochondria (broken line) and on SHAM-resistant NADH oxidase in poky f+ mitochondria (solid line). Concentrations were 0.30 to 1.0 mM NADH, 0.24 to 0.63 mM ADP, and, in the experiment with poky f+, 120 µg of SHAM per ml. Each curve summarizes two experiments, with control rates for wild type of 0.60 to 1.1 amoloms of O per min per mg of protein, and for poky f+ of 0.15 to 0.23 amoloms of O per min per mg of protein. Cyanide was added during State 3, and steady state respiration rates were measured several minutes later.

Fig. 3 (right). Effect of antimycin on NADH oxidase in wild type mitochondria (broken line) and on SHAM-resistant NADH oxidase in poky f+ mitochondria (solid line). Concentrations were 0.50 to 1.0 mM NADH, 0.48 to 0.63 mM ADP, and, in the experiment with poky f+, 120 µg of SHAM per ml. Each curve summarizes two experiments, with control rates for wild type of 1.1 to 1.3 amoloms of O per min per mg of protein, and for poky f+ of 0.14 to 0.22 amoloms of O per min per mg of protein. Antimycin was added during State 1 or State 3. In the former case, 1 to 2 min were allowed before adding substrate, and steady state respiration rates were measured several minutes later; in the latter case, steady state respiration rates were measured several minutes after adding antimycin.

to antimycin, and the fact that more than 90% of the respiration supported by both substrates can be blocked by these inhibitors, suggests that in wild type mitochondria essentially the entire electron flow from NADH and from succinate passes through the same antimycin- and cyanide-sensitive sites.

In poky mitochondria, as expected from whole-cell experiments (6), the situation is more complex. Respiration with pyruvate (plus malate), NADH, or succinate was only partially inhibited by cyanide or antimycin (6 to 50%, depending on the substrate and the age of the mitochondrial preparation); the effects of cyanide and antimycin were not additive (Fig. 4, A and B).

Dual Oxidase System in Poky Mitochondria

We have previously demonstrated that cyanide-resistant respiration in poky cells can be inhibited by salicyl hydroxamic acid (SHAM; Reference 6). Fig. 4, D and E extend this result to poky mitochondria. Addition of cyanide (1.0 mM) to mitochondria respiring with NADH inhibited only 18% of the respiration, but SHAM (120 µg per ml) inhibited the remainder (Fig. 4D). When the order of addition was reversed, SHAM gave partial inhibition (66%) and cyanide inhibited the rest (Fig. 4E). Similar results were obtained during respiration with other substrates, pyruvate (plus malate) or succinate, or when antimycin was substituted for cyanide.

These results, taken together, suggest that the oxidation of NADH and Krebs cycle acids is mediated by two oxidase systems in poky mitochondria; one (previously shown to be a cytochrome system; Reference 5) blocked by 1 mM cyanide or by antimycin, and the other (the alternate oxidase) blocked by SHAM (120 µg per ml). When one oxidase is inhibited, virtually the entire electron flux is forced through the other, a situation that permits the two systems to be studied separately. Some of the properties of the two systems are summarized below.

Activity—In fresh preparations of poky mitochondria respiring with either NADH or pyruvate (plus malate), State 3 rates in the presence of SHAM (120 µg per ml) (taken to represent the cytochrome system) were 0.22 to 0.26 amoloms of O per min per mg of protein, and State 3 rates in the presence of cyanide (1.0 mM) (taken to represent the alternate oxidase) were 0.40 to 0.70 amoloms of O per min per mg of protein. The cytochrome system is therefore one-third to one-half as active as the alternate oxidase in freshly isolated poky mitochondria.

Stability The cytochrome system is more stable, however. In several experiments with NADH as substrate, it lost less than 10% of its activity during 3 to 4 hours after the mitochondria were isolated, while the alternate oxidase lost from 30 to 50% of its activity during the same period. The instability of respiration in isolated poky mitochondria is therefore due mainly to the decreasing activity of the alternate oxidase.

Sensitivity of Alternate Oxidase to SHAM—Fig. 5 illustrates the inhibition of cyanide-resistant respiration (the alternate oxidase) as a function of SHAM concentration, with NADH as substrate. Half-maximal inhibition occurred at 8 µg of SHAM per ml, similar to the value previously found for poky cells (11 µg per ml; Reference 6) and within the range of values reported for higher plant mitochondria (31). Complete inhibition required about 60 µg of SHAM per ml (Fig. 5); at this concentration, SHAM had no effect on the respiration of wild type mitochondria.
FIG. 4. Oxygen electrode recordings of respiration in poky f+ mitochondria. A, B, effect of cyanide and antimycin on NADH oxidase. NADH (0.53 mM), ADP (0.67 mM), cyanide (1.0 mM), antimycin (0.21 µg per mg of protein). M = 0.53 mg of mitochondrial protein. C, effect of cyanide on respiration with TMPD plus ascorbate. TMPD (0.2 mM), ascorbate (10 mM), cyanide (1.0 mM), ADP (0.67 mM). M = 0.72 mg of mitochondrial protein. D, E, effect of cyanide and SHAM on NADH oxidase. NADH (0.50 mM), ADP (0.42 mM), cyanide (1.0 mM), SHAM (120 µg per ml). M = 0.46 mg of mitochondrial protein. In all experiments, the initial O2 concentration was 240 µM, and numbers adjacent to traces are respiration rates in µatoms of O per min per mg of protein.

Sensitivity of Poky Cytochrome System to Cyanide and Antimycin—In parallel experiments, inhibition of SHAM-resistant respiration (the cytochrome system) was determined in poky mitochondria as a function of cyanide and antimycin concentration, again with NADH as substrate (Figs. 2 and 3). Half-maximal inhibition occurred at 0.006 mM cyanide and at 0.044 µg of antimycin per mg of protein, slightly lower than the equivalent concentrations for the wild type cytochrome system 

In all experiments, the initial O2 concentration was 240 µM, and numbers adjacent to traces are respiration rates in µatoms of O per min per mg of protein.

Quantitatively, poky mitochondria contain abnormally low amounts of cytochrome oxidase activity, as indicated by respiration rates with TMPD plus ascorbate, 0.38 and 0.99 µatoms of O per min per mg of protein for poky and wild type, respectively (Table I). (These data in fact underestimate the difference between the two strains, because the concentration of TMPD used in the experiment was not sufficient to saturate the wild type system.) The deficiency of cytochrome oxidase activity in poky correlates well with the previously reported deficiency of cytochrome aa3 (2-5).

Fig. 4C also illustrates an unexpected finding regarding respiration with TMPD plus ascorbate in poky mitochondria; after respiration had been inhibited by 1.0 mM cyanide, the addition of ATP caused a slight stimulation of respiration. ATP did not stimulate respiration in wild type mitochondria under these conditions, and the basis for its effect in poky is not certain. One
possibility is that ADP was converted to ATP by endogenous adenylate kinase, and that this ATP was then used to drive respiration through the cyanide-resistant oxidase by way of reversed electron transport through Site 2; the next section will show that ATP does have this effect.

Reversed Electron Transport from Cytochrome c to Alternate Oxidase

The relationship between the two oxidase systems of poky mitochondria was investigated by means of reversed electron transport, in which energy supplied from ATP is used to make electrons flow through a phosphorylation site against a potential gradient (33-35). The idea was to see whether electrons donated to the c type cytochromes by TMPD plus ascorbate could be made to flow in the reverse direction through Site 2 and then through the cyanide-resistant oxidase. One such experiment is illustrated in Fig. 6A. When ATP was added to a suspension of poky mitochondria in the presence of TMPD plus ascorbate and 1 mM cyanide (to block electron flow through cytochrome oxidase), there was a marked stimulation of respiration. This respiration appeared to be energy-dependent since it was inhibited by CCCP at uncoupling concentrations (Fig. 6B; and see next paper) and by oligomycin (which prevents the generation of high energy intermediates from ATP; 35).

The effects of respiratory inhibitors on the ATP-stimulated respiration were examined in an attempt to define the path of electron flow. Antimycin and SHAM were found to be effective inhibitors (Fig. 6, C and E) at concentrations similar to those required for inhibition of NADH oxidase in poky mitochondria; half-maximal inhibition of the ATP-stimulated respiration occurred at 0.060 μg of antimycin per mg of protein and 10 μg of SHAM per ml (data not shown), and half-maximal inhibition of NADH oxidase at 0.044 μg of antimycin per mg of protein (Fig. 3) and 8 μg of SHAM per ml (Fig. 5). Results with these two inhibitors were therefore consistent with the idea that electrons were passing through the b cytochromes and the cyanide-resistant oxidase. (Pyrrolnitrin also blocked ATP-stimulated respiration (Fig. 6D) but it could have been acting as an uncoupler rather than as an oxidation-reduction inhibitor; therefore, the pyrrolnitrin results do not reveal whether there was electron flow through the flavin region.)

**DISCUSSION**

Respiratory System of Wild Type Neurospora—The results presented here and in preceding papers (5, 6) indicate that, overall, the respiratory system of wild type *Neurospora* is quite similar to that of mammalian mitochondria.

The electron transport components include pyridine nucleotides (mostly NADH), flavoproteins, two b cytochromes (b6, a soluble cytochrome c (~543, a membrane-bound cytochrome c), and cytochromeaa3 (5). The absorption maxima of the cytochrome components differ by only a few nanometers from those of the corresponding components in animal mitochondria (5, 36).

*Neurospora* mitochondria respire with several Krebs cycle acids, with TMPD plus ascorbate, and also (unlike mammalian mitochondria but like mitochondria isolated from higher plants, yeast, and other filamentous fungi; 37-42) with exogenous NADH. The recent results of Weiss et al. (36) suggest that exogenous and endogenous pyridine nucleotides enter the respiratory chain of *Neurospora* by way of separate dehydrogenases.

The classical respiratory inhibitors cyanide and antimycin (and, under the appropriate experimental conditions, pyrrolnitrin) have similar effects in *Neurospora* and mammalian mitochondria. For rotenone and amytal, the situation is more complex. In the present experiments both compounds, at concentrations that are maximally effective in animal and yeast mitochondria (20, 25), did not have a substantial effect on respiration with NADH-linked substrates in *Neurospora*.

Weiss et al. (36), however, reported significant inhibition by rotenone in their preparations of *Neurospora* mitochondria. The
reason for the discrepancy between the two sets of results is not clear, but experiments with yeast have shown that rotenone sensitivity may be gained or lost as a result of changes in the growth conditions, medium, aeration, or phase of growth (25, 43, 44). A similar situation may exist in Neurospora, a related ascomycete.

Respiratory System of Poky—The poky mutant is well known to be deficient in membrane-bound cytochromes (including the two b cytochromes, cytochrome c550, and cytochrome aa3; Reference 5) and to respire, in part, by a pathway that is insensitive to antimycin, cyanide, and azide (6-8). A primary aim of the present experiments was to characterize the differences between wild type and poky in more detail by comparing mitochondrial respiration in the two strains.

The two types of mitochondria respire with the same range of substrates, but State 3 rates for poky are uniformly 20 to 40% lower than for wild type (Table I). If one can assume that State 3 respiration is limited by components of lower potential than the terminal oxidase systems (45), the lower State 3 rates suggest that poky mitochondria are deficient in dehydrogenases or in some component of the flavin region.

The early portions of the respiratory chain appear qualitatively normal in poky. Both for pyruvate (plus malate) and for NADH, the K1/2 values in poky and wild type mitochondria are nearly equal, suggesting that the two strains have the same mitochondrial transport systems and dehydrogenases or both for these two substrates. Succinate may be an exception, since the K1/2 is somewhat higher in poky than in wild type (Table II). In fact both types of mitochondria show higher K1/2 values than have been reported for succinate dehydrogenases from animal tissues (ca. 0.5 mM; references 46-48) raising the possibility that the transport of succinate is rate-limiting in Neurospora mitochondria or that the mitochondria contain significant concentrations of a competitive inhibitor of succinate dehydrogenase (e.g., oxaloacetate).

The flavin regions of wild type and poky mitochondria cannot be compared in detail on the basis of the present results since the traditional inhibitors, rotenone and amytal, were ineffective. Pyrrolnitrin, which has been reported to inhibit electron transport in the flavin region of animal and yeast mitochondria (26, 27), gave similar inhibition curves for wild type and poky, suggesting that at least one component of the flavin region is common to both strains.

The major difference between wild type and poky mitochondria is in their oxidase systems. Poky contains both a cytochrome chain, sensitive to cyanide and antimycin (Figs. 2 and 3), and an alternate, cyanide- and antimycin-resistant oxidase which can be specifically inhibited by SHAM (Figs. 4D and 5). The alternate oxidase is about 2 to 3 times as active as the cytochrome system in poky mitochondria (and also in poky cells; reference 6).

Lack of Participation of Cytochrome Components in Cyanide-resistant Respiration—The role of the cytochromes in the alternate, cyanide-resistant pathway of poky mitochondria was investigated in a previous spectrophotometric study (5) by means of inhibitors (cyanide and antimycin on the one hand, SHAM on the other hand), making use of the principle that electron transport components on the substrate side of a site of inhibition should become completely reduced when the inhibitor is added. Antimycin and cyanide, which inhibit less than 50% of the respiration of poky mitochondria, caused nearly complete reduction of the b and c type cytochromes, respectively, indicating that neither b nor c cytochromes are obligatory components of the alternate oxidase system. In addition SHAM, at concentrations that block virtually all respiration through the alternate oxidase, had little effect on the oxidation-reduction level of cytochrome c, confirming the lack of interaction between cytochrome c and the alternate oxidase.

A more quantitative approach to this question has now been made using TMPD plus ascorbate to donate electrons directly to the c cytochromes. In poky mitochondria as in wild type, nearly all of the respiration with TMPD plus ascorbate is inhibited by 1 mM cyanide (Figs. 4C and 6A). The residual respiration rate, ca. 0.02 μatoms of O per min per mg of protein (corrected for a contribution from the nonenzymatic oxidation of TMPD plus ascorbate), is the maximal rate of electron transfer that could occur from cytochrome c to the cyanide-resistant alternate oxidase. This rate is clearly too low (compared with the rate of cyanide-resistant respiration, ca. 0.50 μatoms of O per min per mg of protein with NADH as substrate) for cytochrome c to be a kinetically important component in the alternate oxidase pathway.

Evidence for a Branch Point Located in Flavin Region—Having ruled out the idea that the alternate oxidase interacts with the cytochromes, we must still consider its relationship to the initial segment (dehydrogenases, flavoproteins) of the regular respiratory chain. One possibility is that the alternate oxidase is completely separate, as depicted in Fig. 7A (parallel model). Alternatively it and the cytochromes may share dehydrogenases and

![Fig. 7. Possible models for the poky respiratory system. A, parallel electron transport systems. B, branched electron transport system. X depicts the cyanide-resistant oxidase and Y an unspecified component which can transfer electrons from the flavin region to the cyanide-resistant oxidase or to the b type cytochromes. Inhibition sites are indicated by dashed lines. The cytochrome components are those described by Lambowitz et al (5).](http://www.jbc.org/)
flavoproteins, thereby forming a branched system; a model
of this sort (Fig. 7B) has been proposed to account for cyanide-
resistant respiration in higher plant mitochondria (31, 49–53).
Although the results presented in this paper do not permit an
unambiguous choice between the two models, two sets of data
give relevant information.

The respiratory rate of poky mitochondria with NADH and
Krebs cycle acids is almost always less than would be predicted
from the sum of the cyanide-resistant and SHAM-resistant rates
measured separately. In the traces shown in Fig. 4, D and E,
for example, the respiratory rate in the absence of inhibitor was
16% less than the sum of the cyanide- and SHAM-resistant rates;
in other experiments the uninhibited respiratory rate was
as much as 40% less than the sum of the two oxidase activities.
These results imply that one (or both) of the oxidase systems
does not operate at maximal activity in the absence of inhibitor
(also see next paper) and suggest that the two systems compete
for reducing equivalents at some point following the rate-limiting
step in the over-all respiration process. If substrate penetration
into the mitochondria is rate-limiting, either of the models in
Fig. 7 would be possible; if, on the other hand, the dehydro-
genases or flavoproteins are rate-limiting, only a branched model
would fit the data.

Additional information, more useful in distinguishing between
the parallel and branched models, comes from the demonstration
that the cyanide-resistant oxidase can be reduced by cytochrome
C in an energy-dependent reaction (Fig. 6). Presumably this
reaction involves the reversal of electron transport along all or
part of the chain from cytochrome C toward NADH, the extent of
the chain involved being dependent on the location of the branch
point (if any) and on the location and efficiency of the
phosphorylation sites. In the next paper it will be shown that
Site 1 in poky mitochondria is either extremely inefficient (effi-
ciency less than 5%, judged from the difference in ADP/O ratios
with NADH-linked substrates and with succinate) or perhaps
absent altogether. Consequently there is no direct way to
supply energy for electron transport from the flavin region to
NADH, and it is reasonable to suppose that reversed electron
flow between these points occurs very slowly if at all. If this is
the case, the observed rapid rate of respiration from cytochrome
C through the cyanide-resistant oxidase requires that there be a
direct link between the two systems on the oxygen side of the
dehydrogenases (as shown in Fig. 7B).

Further experiments will be required to test the assumptions
made in this argument, and also to identify the alternate oxidase
and the components of the branch point.

Acknowledgments—The authors thank Drs. Jui H. Wang,
Walter D. Bonner, and Clifford L. Slayman for their valuable
discussions and criticism.

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