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# Cyanide-Resistant Respiration in *Neurospora crassa*

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Cell respiration in wild type and *poky* was studied as part of a long-term investigation of cyanide-resistant respiration in *Neurospora*. Respiration in wild type proceeds via a cytochrome chain which is similar to that of higher organisms; it is sensitive to antimycin A or cyanide. *Poky*, on the other hand, respires by means of two alternative oxidase systems. One of these is analogous to the wild-type cytochrome chain in that it can be inhibited by antimycin A or cyanide; this system accounts for as much as 15% of the respiration of *poky* $f^-$ and 34% of the respiration of *poky* $f^+$. The second oxidase system is unaffected by antimycin A or cyanide at concentrations which inhibit the cytochrome chain maximally. It can, however, be specifically inhibited by salicyl hydroxamic acid. The cyanide-resistant oxidase is not exclusive to *poky*, but is also present in small quantities in wild type grown under ordinary circumstances. These quantities may be greatly increased (as much as 20-fold) by growing wild type in the presence of antimycin A, cyanide, or chloramphenicol.

The respiratory chain of the fungus *Neurospora crassa* is similar in many respects to that of higher organisms. Mitochondria isolated from wild-type *Neurospora* show well defined absorption peaks corresponding to *b*-, *c*-, and *a*-type cytochromes (8, 12, 18, 30; Lambowitz et al., *submitted for publication*); and electron transport is inhibited by antimycin A (7, 8, 30), acting between cytochromes *b* and *c*, and by cyanide or azide (4, 7, 8, 28, 30), acting at cytochrome $aa_3$.

During the past 20 years, a number of *Neurospora* mutants have been isolated with abnormal respiratory systems (2, 3, 6, 13, 15, 17, 23, 27). The most extensively studied mutant strain has been *poky* or *mi-1*, which lacks almost completely the absorption bands corresponding to the *a*- and *b*-type cytochromes but contains a marked excess of cytochrome *c* (9, 17, 18; Bonner et al., *manuscript in preparation*). Since *Neurospora* is an obligate aerobe, presumably depending on the respiratory chain for the oxidation of reducing equivalents and the synthesis of adenosine triphosphate, there has been considerable interest in the fact that mutants such as *poky* are viable. In 1953, Tissières et al. (28) showed that *poky* mycelia do respire in spite of the apparent absence of the usual terminal oxidase (cytochrome $aa_3$). The respiration of *poky* is conspicuously different from that of wild-type *Neurospora*, however, in being resistant to cyanide or azide (4, 28). To account for these findings, Tissières et al. postulated that *poky* possesses an alternate oxidase which is not affected by cyanide or azide. The identity of the postulated oxidase and its position in the electron transport chain remain unknown.

We have undertaken a study of cyanide-resistant respiration in *Neurospora*, with the long-range goal of identifying the components of the pathway and establishing their relationship to the standard cytochrome chain. The present work considers some characteristics of cell respiration in wild type and *poky* and is intended as background for subsequent investigations of the cyanide-resistant pathway in isolated mitochondria. The evidence presented is compatible with the idea that both wild type and *poky* utilize branched electron transport systems, in which one branch is the standard cytochrome chain and the other branch is an alternate cyanide-insensitive oxidase. Although in the wild-type strain the cyanide-resistant branch accounts for less than 10% of total respiration, we find that its activity can be increased as much as 20-fold by growing the cells in the presence of antimycin A, cyanide, or chloramphenicol.

## MATERIALS AND METHODS

**Strains of Neurospora.** The wild-type strain, RL21a, was derived from the original Lindegren wild types

L1A and L25a (22). The *poky* strains NSX f⁺a (18) and NSX f⁻a were obtained from Rifkin and Luck; f⁺ is a nuclear gene suppressor of the *poky* mutation which increases the growth rate but does not restore the wild-type respiratory system (16). Procedures for maintaining strains and preparing conidia were described previously (22).

**Growth of cells.** Cells were grown as shaken liquid cultures at 25 C in Vogel's minimal medium (29) plus 2% sucrose (160 ml of medium per 1-liter Florence flask). Under these conditions, all three strains displayed exponential growth with doubling times of 2 to 3 hr for the wild type, 3.5 to 4.5 hr for *poky* f⁺, and 5.5 to 6.5 hr for *poky* f⁻ (see Fig. 1).

**Respiration measurements.** Respiration was measured polarigraphically as described by Slayman and Slayman (21). The reaction was begun by pipetting about 0.5 ml of a freshly prepared, aerated cell suspension (10 mg dry wt/ml; *see below*) into a reaction chamber containing 18.5 ml of respiration medium: Vogel's minimal medium (29) plus 2% glucose, *p*H 5.8, saturated with air (i.e., containing 240 μM oxygen). Glucose rather than sucrose was used in the respiration medium to ensure that substrate was not rate-limiting; the β-fructofuranosidase (invertase) of *Neurospora* is extracellular, and a portion of the enzyme is known to be lost when conidia are washed (14). The reaction chamber was maintained at 25 C, and the suspension was mixed by a magnetic stirring bar. At the end of each measurement, cells were harvested quantitatively from the chamber, rinsed, dried, and weighed. Results were expressed as microliters of $O_2$ consumed per hour per milligram (dry weight). Correction was made for back diffusion of oxygen through the injection port.

Cell suspensions for the respiration measurements were prepared by harvesting cells from several culture flasks on a filter (Millipore Corp.; pore size, 1.2 μm) and resuspending the pellet in a small volume of respiration medium. During an experiment, the stock suspension was kept aerobic by bubbling with $CO_2$-free air. Respiration measurements were performed within 90 min after harvesting, and control rates were checked frequently.

**Inhibitors.** Antimycin A (Sigma), chloramphenicol (Sigma), and salicyl hydroxamic acid (SHAM; Aldrich) were dissolved in absolute ethanol and prepared fresh for each experiment. NaCN (Baker) was made up in aqueous solution at *p*H 7.0; fresh solutions were used for all experiments in which respiration was measured as a function of cyanide concentration, and solutions which had been stored tightly stoppered at 0 C for not more than 3 days were used for routine assays of "cyanide-insensitive respiration" (respiration not inhibited by 1 mM $CN^-$). Because of the volatility of HCN, stock solutions of cyanide were assayed by titration to insure that the initial concentrations were accurate. Titration also revealed that the cyanide concentration decreased less than 5% in 3-day refrigerated solutions.

## RESULTS

**Respiration rates of wild type and poky.** Tissières et al. (28) reported respiration rates of 68 to 76 μliters of $O_2$ per hr per mg dry wt for wild-type *Neurospora* (grown in aerated liquid culture for 8 and 26 hr) and 22 to 33 μliters of $O_2$ per hr per mg dry wt for *poky* f⁻ (grown under the same conditions for 40 to 168 hr). However, it was not established that either strain was in exponential phase.

Preliminary experiments in our laboratory indicated considerably higher respiration rates for carefully controlled log-phase cultures of both wild type and *poky*, and it therefore seemed worthwhile to carry out a systematic investigation of respiration rates as a function of time throughout exponential phase. The results of this investigation are summarized in Fig. 1 (i). During exponential phase, the mean respiration rates of both strains of *poky* (approximately 200 μliters of $O_2$ per hr per mg dry wt) were about twice the mean rate for wild-type *Neurospora* (100 μliters per hr per mg dry wt), and about 8 times the rates reported for *poky* by Tissières et al. (ii). Whereas the respiration of *poky* was fairly constant throughout exponential phase, wild-type respiration declined slowly from a peak of 100 μliters of $O_2$ per hr per mg dry wt at 6 hr to 75 μliters of $O_2$ per hr per mg. dry wt at 16 hr. It is not clear whether this decline, which was seen repeatedly, reflected a true change in respiratory metabolism or whether—because oxygen consumption was measured with respect to the dry weight of the cells—some other cell component (e.g., cell wall) may have increased during exponential phase in the wild-type strain.

In the experiments to be described, all three strains were studied at or near the maximal level of respiration, during mid-log phase. Although control respiration rates did not vary by more than 5 to 10% during a single experiment, there were sometimes larger variations (up to 30%) from one experiment to another. Therefore, most experimental results have been plotted as per cent of control respiration, but actual control rates are included in the legends.

**Dependence of respiration rate on oxygen concentration.** It was important to insure that respiration measurements for both strains were carried out under conditions where oxygen was not rate-limiting. From the results of Tissières et al. (28), there was reason to suspect that *poky* respiration might show an altered oxygen dependence, since an increased rate was observed when pure oxygen was substituted for air. For oxygen concentrations below 240 μM, we found a constant rate of respiration down to 50 μM $O_2$ for *poky* (Fig. 2a), and down to 5 μM $O_2$ for wild type. All respiration measurements reported below were carried out within these limits.

**Effect of cyanide and antimycin A on respiration.** As expected, nearly all of the respiration of

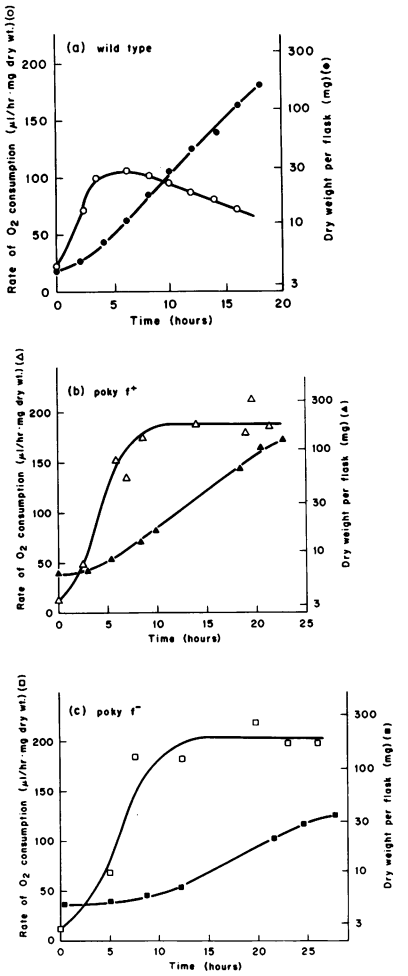


FIG. 1. *Growth and respiration rate as a function of time. a, Wild type (respiration data averaged from three experiments with average SEM 15%, maximal SEM 30%); b, poky f+ (data from one experiment); and c, poky f- (respiration data from two experiments). Filled symbols indicate dry weight per flask in milligrams; open symbols indicate the rate of $O_2$ consumption in microliters of $O_2$ per hour per milligram (dry weight).*

wild-type *Neurospora* was inhibited by low concentrations of cyanide (Fig. 3) or antimycin A (Fig. 4) (half-maximal inhibition at 0.012 mM cyanide or 0.014 μg of antimycin A per mg dry wt of cells). These results are consistent with the idea that the bulk of wild-type respiration proceeds by way of the cytochrome pathway.

*Poky* respiration, on the other hand, was unaffected by cyanide or antimycin A at concentrations which inhibited wild type maximally (Fig. 3 and 4). Although cyanide at very high concentrations did inhibit *poky* respiration, it could well

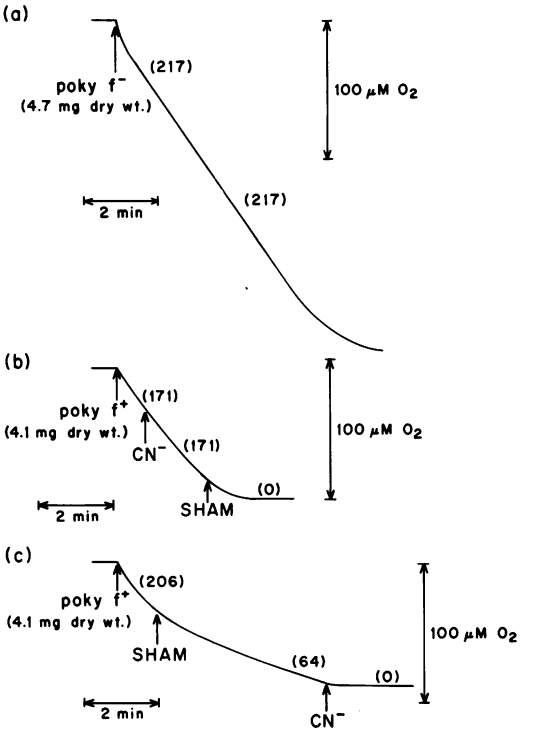


FIG. 2. *Oxygen electrode recordings of respiration of poky. a, Control trace for poky f-; b, addition of cyanide (1.0 mM) followed by salicyl hydroxamic acid (SHAM; 120 μg/ml) to poky f+; c, addition of SHAM (120 μg/ml) followed by cyanide (1.0 mM) to poky f+. In all cases, numbers in parentheses give the respiration rate in microliters of $O_2$ per hour per milligram (dry weight). Slight irregularities at the beginnings of some of the traces are mixing artifacts resulting from injection of the cell suspension into the chamber.*

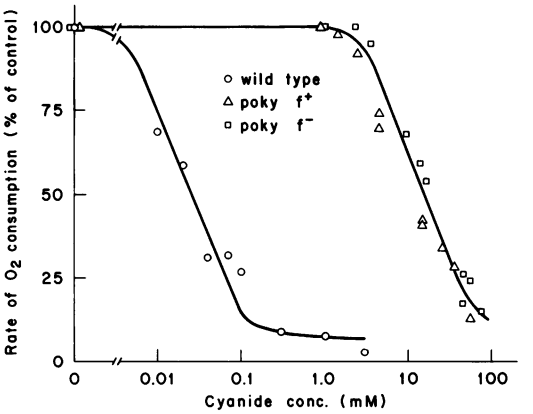


FIG. 3. *Effect of cyanide on the respiration of wild type, poky f+, and poky f-. Control rates were 98 μliters of $O_2$ per hr per mg dry wt for wild type; 175 and 208 μliters of $O_2$ per hr per mg dry wt for poky f+ (two independent experiments); and 174 ± 17 μliters of $O_2$ per hr per mg dry wt for poky f- (mean ± SEM; four independent experiments).*

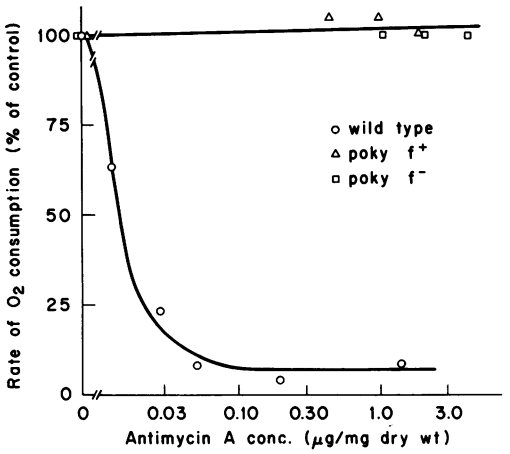


FIG. 4. *Effect of antimycin A on the respiration of wild type, poky f⁺, and poky f⁻. Control rates were 90 and 108 μliters of $O_2$ per hr per mg dry wt for wild type (two independent experiments); 154 μliters of $O_2$ per hr per mg dry wt for poky f⁺; and 168 μliters of $O_2$ per hr per mg dry wt for poky f⁻.*

have been acting at a site other than the terminal oxidase.

**Inhibition of cyanide-resistant respiration by SHAM.** Recently, Schonbaum et al. (19) showed that hydroxamic acids are specific inhibitors of the cyanide-insensitive oxidase found in the mitochondria of some higher plants. Experiments with these inhibitors have demonstrated that mitochondria from mung beans and skunk cabbages respire via a branched electron transport pathway, involving both a cytochrome chain and an alternate oxidase which contacts the chain in the flavoprotein region (5, 19). In such mitochondria, when cyanide or antimycin A is present to inhibit the cytochrome system, the electron flux is carried by the alternate oxidase, and respiration becomes sensitive to hydroxamic acids. Conversely, when hydroxamic acids are present initially, the electron flux is carried by the cytochrome system, and respiration becomes sensitive to cyanide or antimycin A.

The traces presented in Fig. 2b and c indicate that a similar situation may exist in *poky*. Figure 2b shows the effect of sequential addition of cyanide (1.0 mM) and SHAM (120 μg/ml) to a respiring suspension of *poky* f⁺ cells. The addition of cyanide had no visible effect. However, nearly all of the cyanide-resistant respiration was inhibited by subsequent addition of SHAM. Fig. 2c shows that when SHAM was added first, at a sufficient concentration to block the alternate oxidase, about 70% of the original respiration was inhibited. The remaining SHAM-resistant respiration was inhibited by 1.0 mM cyanide (or, in other experiments, by antimycin A). These results are consistent with the idea that *poky* utilizes a branched electron transport pathway similar to the one described for mung beans and skunk cabbages (1, 5, 19, 24, 25, 26). Apparently, *poky* has a sufficient quantity of the alternate oxidase to carry the entire electron flux when the cytochrome system is blocked by cyanide; however, the cytochrome system can carry only a fraction of the original electron flux when the alternate oxidase is blocked by SHAM.

Experiments were next carried out to characterize each branch of the system with respect to sensitivity to inhibitors.

(i) Titration of cyanide-resistant respiration in *poky* with SHAM revealed half-maximal inhibition occurring at 11 μg/ml (0.072 mM) (Fig. 5). This is within the range of concentrations required for half-maximal inhibition of cyanide-insensitive succinic acid oxidase activity in higher plant mitochondria (19). Figure 5 also shows that the concentration of SHAM required for half-maximal inhibition is not affected by the state of the f gene.

(ii) To test the idea that SHAM-resistant respiration proceeds via a cytochrome system, we examined its sensitivity to cyanide (Fig. 6) and to antimycin A (Fig. 7). (Because of the small amount of SHAM-resistant respiration in *poky* f⁻, these experiments were performed with *poky* f⁺ only.) Low concentrations of both inhibitors were effective. Quantitatively, the pathway is similar to the wild-type cytochrome system in its sensitivity to cyanide (half-maximal inhibition at

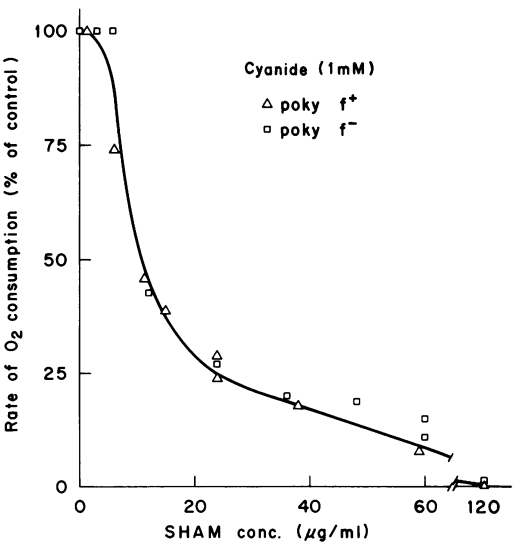


FIG. 5. *Effect of salicyl hydroxamic acid (SHAM) on cyanide-resistant respiration in poky f⁺ and poky f⁻. All measurements were made in the presence of 1.0 mM cyanide. Control rates were 166 to 221 μliters of $O_2$ per hr per mg dry wt for poky f⁺ and 174 to 300 μliters of $O_2$ per hr per mg dry wt for poky f⁻.*

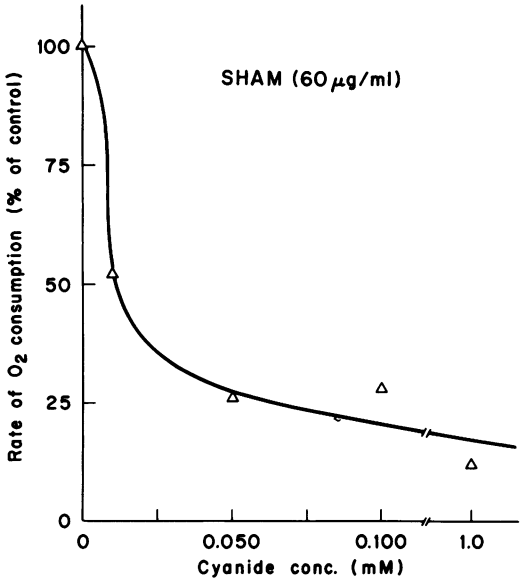


FIG. 6. *Effect of cyanide on salicyl hydroxamic acid (SHAM)-resistant respiration in poky f⁺. All measurements were made in the presence of 60 μg of SHAM per ml. Control rates were 60 to 93 μliters of $O_2$ per hr per mg dry wt.*

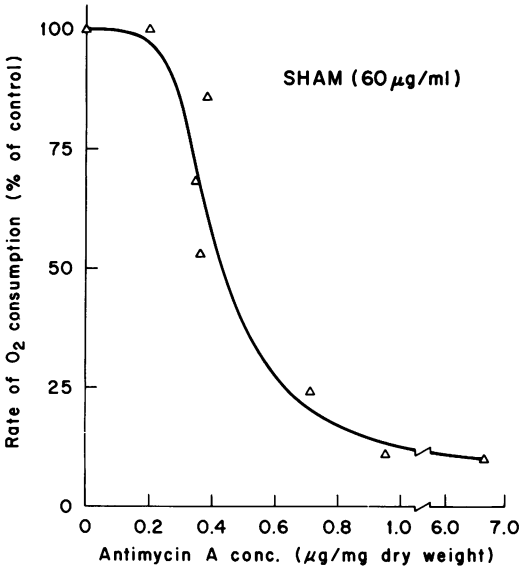


FIG. 7. *Effect of antimycin A on salicyl hydroxamic acid (SHAM)-resistant respiration in poky f⁺. All measurements were made in the presence of 60 μg of SHAM/ml. Control rates were 56 to 92 μliters of $O_2$ per hr per mg dry wt.*

0.009 mM compared to 0.012 mM for wild type). However, it is about 30 times less sensitive to antimycin A (half-maximal inhibition at 0.42 μg/mg dry wt of cells compared to 0.014 μg/mg dry wt of cells for wild type). The reason for this discrepancy is not known. However, the following control experiments rule out any complicating interaction between SHAM and antimycin A. (i) SHAM (120 μg/ml) did not cause a comparable decrease in the sensitivity of the wild-type cytochrome system to antimycin A. (ii) Even though SHAM-resistant respiration in *poky* was not completely inhibited by low concentrations of antimycin A, the remaining respiration was fully sensitive to 1 mM cyanide, indicating that antimycin A had not impaired the effectiveness of SHAM. The experiments described in this section, taken together, suggest that SHAM-resistant respiration utilizes cytochrome components, but that these may have an altered affinity for antimycin A.

The fraction of *poky* respiration which is resistant to high concentrations of SHAM is a measure of the maximal activity of the cytochrome system. Figure 8 shows that this activity is greater in *poky* f⁺ than in *poky* f⁻, at least in the case of mid-log phase cells [62 ± 13 μliters of $O_2$ per hr per mg dry wt and 34 ± 11 μliters of $O_2$ per hr per mg dry wt, respectively (mean ± SD; difference significant at level of $P \simeq 0.025$)]. Figure 8 also shows that SHAM does not inhibit wild-type respiration at concentrations which inhibit *poky* maximally.

**Stimulation of cyanide-resistant respiration in wild type by antimycin A.** Having established that a substantial portion of *poky* respiration occurs

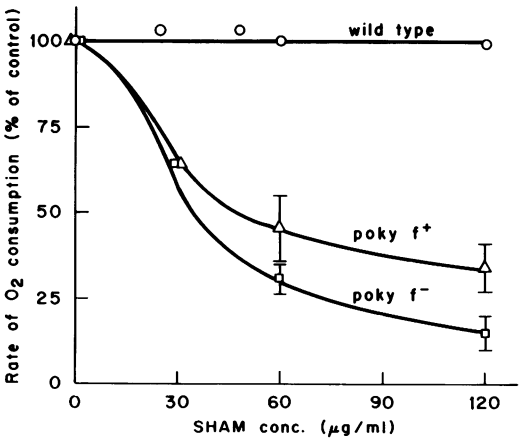


FIG. 8. *Effect of salicyl hydroxamic acid (SHAM) on the respiration of wild type, poky f⁺, and poky f⁻. Control rates were 68 and 100 μliters of $O_2$ per hr per mg dry wt for wild type (two independent experiments) and 183 ± 5 and 224 ± 10 μliters of $O_2$ per hr per mg dry wt for poky f⁺ and poky f⁻ (mean ± SEM; 8 independent determinations for poky f⁺ and 10 independent determinations for poky f⁻). The error limits in the figures depict standard deviations from the mean of 3 or more values.*

by way of a cyanide-resistant pathway, we next explored the question of whether this pathway is unique to *poky*, or whether wild-type *Neurospora* also has the capacity to develop it under some circumstances. Preliminary experiments suggested that wild-type cells, even under normal growth conditions, might possess a small amount of the pathway. Nearly all of the respiration resistant to 1 mM cyanide (about 8% of the total in 10-hr wild-type cells) appeared to be inhibited by SHAM. These experiments were complicated by the very low levels of respiration being measured, however, and it seemed worthwhile to look for experimental conditions which would stimulate cyanide-resistant respiration in the wild-type strain. Three approaches were tried: blocking the cytochrome pathway of growing cells with antimycin A, or with cyanide, or growing the cells in the presence of chloramphenicol to inhibit mitochondrial protein synthesis.

Figure 9 shows that antimycin A did indeed cause a dramatic stimulation of cyanide-resistant respiration in early exponential-phase wild-type cells. Conidia were inoculated into minimal medium and allowed to grow until the beginning of exponential phase (6 hr in this experiment). Antimycin A was then added to a final concentration of 0.05 μg/ml (1.2 μg of antimycin A per mg dry wt of cells), sufficient to inhibit respiration maximally (see Fig. 4). Growth stopped abruptly, and during the following 12 to 13 hr there was a steady increase in cyanide-resistant respiration to a peak of 90 μliters of $O_2$ per hr per mg dry wt. In other experiments, not shown, even higher rates of cyanide-resistant respiration (up to 180 μliters of $O_2$ per hr per mg dry wt) were attained. The peak rate was maintained for a relatively short time (2 to 3 hr). Subsequently, the total $Q(O_2)$ remained high, but respiration became progressively more sensitive to cyanide, and at the same time the cells began to grow again (Fig. 9).

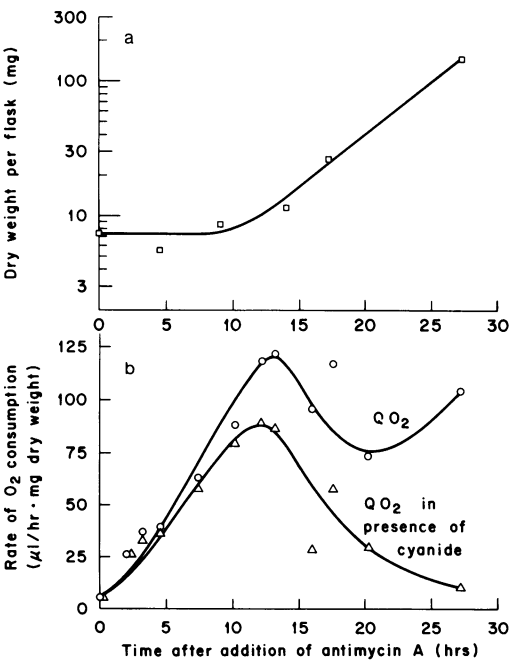


FIG. 9. *Effect of antimycin A on the growth (a) and respiration (b) of early exponential-phase cells of wild-type Neurospora. Antimycin A (final concentration, 0.05 μg/ml) was added at zero time, 6 hr after inoculation of the culture. At that time, the cells had not yet completed one doubling, and the cell density was 0.043 mg dry wt/ml.*

Experiments were carried out to define optimal conditions for the stimulation of cyanide-resistant respiration by antimycin A.

(i) The maximal activity reached by the cyanide-resistant pathway did not vary significantly with the titer of antimycin A over the range 0.69 to 76 μg of antimycin A per mg dry wt of cells, but the higher concentrations of antimycin A did delay the return of cyanide-sensitive respiration and the resumption of growth. There was some indication that the return of cyanide-sensitive respiration and the resumption of growth reflected an increased resistance to antimycin A since they were only partially prevented by a second dose of the drug (Fig. 10). The mechanism of this adaptation is being investigated.

(ii) Somewhat surprisingly, the ability of wild-type cells to develop cyanide-resistant respiration was found to decline rapidly after early exponential phase. As shown in Table 1, the addition of antimycin A at 5.5 hr caused only 25% as much stimulation as addition at 0 or 3 hr; and addition at 10.5 hr—still the middle of exponential phase—had a barely detectable stimulatory effect. The reason for the age dependence of antimycin A stimulation is unknown.

(iii) Finally, solvent controls showed that ethanol (in which the antimycin A was routinely dissolved) was not itself capable of stimulating cyanide-resistant respiration, nor (at concentrations below 0.6%) did it inhibit the stimulation by antimycin A (Table 2).

**Characteristics of cyanide-resistant respiration in wild type.** Further studies of cyanide-resistant respiration in wild type were performed on early exponential-phase cells which had been incubated in antimycin A (0.05 μg/ml) for 12 hr. Respiration in these cells was found to be similar to *poky* respiration in the following respects. (i) Oxygen consumption was resistant to antimycin A as well as to cyanide. In one experiment, for example, 63% of the respiration remained after

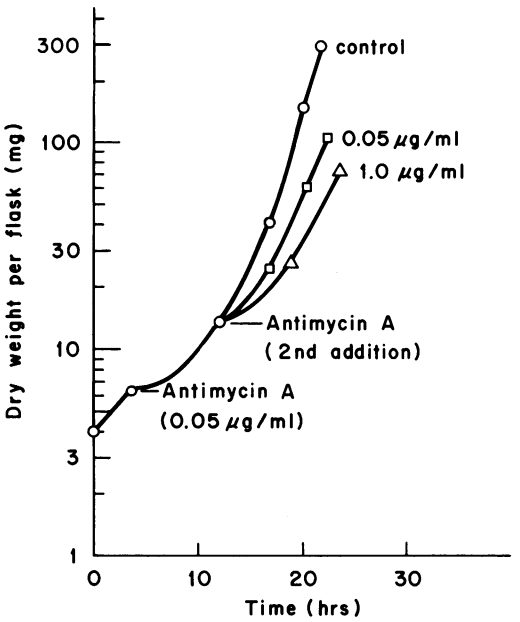


FIG. 10. *Effect of two successive additions of antimycin A on wild-type Neurospora. First addition (0.05 μg/ml) at the beginning of exponential phase (3.5 hr); second addition (0, 0.05, or 1.0 μg/ml) at 12 hr. Parallel respiration measurements showed 72% of the respiration to be resistant to cyanide (1.0 mM) at 12 hr, just before the second addition of antimycin A. At the end of the experiment there was no cyanide-resistant respiration in the control, 24% in cells which had received a second dose of 0.05 μg of antimycin A per ml, and 59% in cells with a second dose of 1.0 μg of antimycin A per ml.*

TABLE 1. *Effect of cell age on the ability of wild-type Neurospora to develop cyanide-resistant respiration in response to antimycin A*[a]

| Age of cells (hr) | Rate of respiration before addition of antimycin A[b] | Maximal rate of respiration insensitive to 1 mM cyanide[b] |
|---|---|---|
| Conidia | 21 | 143 |
| 3 | 96 | 146 |
| 5.5 | 131 | 33 |
| 10.5 | 106 | 6 |

[a] Cells were harvested on sterile filters (Millipore; pore size, 1.2 μm) and resuspended in fresh culture flasks at a density of 0.024 to 0.034 mg dry wt per ml. Antimycin A (final concentration, 0.05 μg/ml) was added to each flask, and the cultures were incubated. Respiration was measured at approximately 6, 12, and 14 hr after the addition of antimycin A.

[b] Expressed as microliters of $O_2$ per hour per milligram (dry weight) of cells.

the addition of 3.3 μg of antimycin A per mg (dry wt) of cells, and 77% remained after the addition of 1 mM cyanide. (ii) The antimycin A- and cyanide-resistant pathway was almost completely inhibited by SHAM (60 μg/ml), and the sensitivity to SHAM was similar to that of *poky* (Fig. 11). Taken together, these results suggest that cyanide-resistant respiration is mediated by the same oxidase in both wild type and *poky*.

TABLE 2. *Effect of ethanol on the development of cyanide-resistant respiration by wild-type Neurospora in response to antimycin A*[a]

| Ethanol concn (%) | Antimycin A concn (μg/ml) | Maximal rate of respiration insensitive to 1 mM $CN^-$[b] |
|---|---|---|
| 0.3 | | 9 |
| 1.9 | | 8 |
| 0.3 | 0.05 | 175 |
| 0.6 | 0.05 | 159 |
| 1.5 | 0.05 | 33 |
| 1.9 | 0.05 | 24 |

[a] Antimycin A and/or ethanol was added to cultures of wild-type *Neurospora* after 3 hr of growth. Respiration measurements were made at 4- to 6-hr intervals and were continued for 8 hr in the ethanol controls, or until the resumption of cyanide-sensitive respiration (12 to 23 hr) in the antimycin A experiments. By comparison, 10-hr wild-type cells typically respire at a rate of 10 μliters of $O_2$ per hr per mg dry wt in the presence of 1 mM $CN^-$.

[b] Expressed as microliters of $O_2$ per hour per milligram (dry weight) of cells.

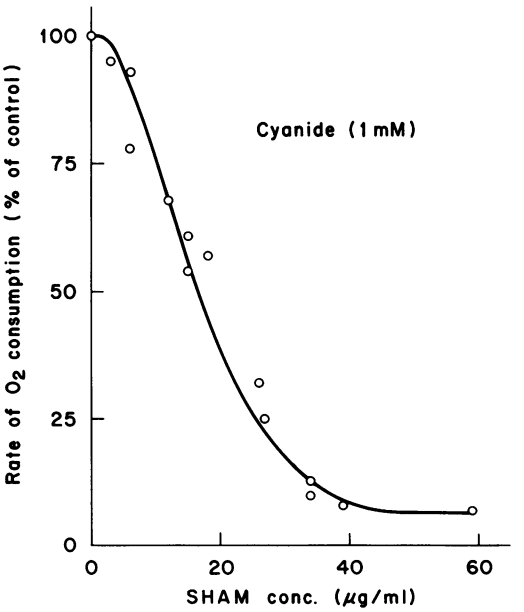


FIG. 11. *Effect of salicyl hydroxamic acid (SHAM) on cyanide-resistant respiration in wild-type cells grown for 12 hr in the presence of antimycin A (0.05 μg/ml). All measurements were made in the presence of 1.0 mM cyanide. Data from two experiments.*

**Stimulation of cyanide-resistant respiration in wild type by cyanide and chloramphenicol.** One might predict, from the antimycin A results, that other inhibitors which block the cytochrome pathway of *Neurospora* would be effective in stimulating the development of the alternate respiratory pathway. As shown in Table 3, this was in fact the case for cyanide, although the maximal stimulated rate was not as great as with antimycin A. Further, the amount of stimulation declined as the cyanide concentration was increased from 0.09 to 0.50 mM.

The third method for stimulating cyanide-resistant respiration in wild-type *Neurospora* had a somewhat different rationale. Rifkin (Ph.D. Thesis, Rockefeller Univ., New York, N.Y., 1969) reported that wild-type cells grown in the presence of chloramphenicol are deficient in cytochromes *a* and *b*, but respire at the normal rate. In this respect, chloramphenicol-grown wild-type cells resemble *poky*, and it seemed likely that their respiration would also be resistant to cyanide. We have confirmed that this is the case. In two experiments where chloramphenicol (2.0 mg/ml) was added during early exponential phase, the growth rate declined (the doubling time increasing from 2 to 5 hr); and, at the same time, there was an increase in cyanide-resistant respiration from 10 μliters of $O_2$ per hr per mg dry wt to a maximal value of about 130 μliters of $O_2$ per hr per mg dry wt within 6 hr after the addition of the inhibitor. This cyanide-resistant respiration was more than 95% blocked by SHAM (120 μg/ml), as expected.

The ability of chloramphenicol to stimulate the development of cyanide-resistant respiration was found to be somewhat less dependent on the age of the culture than was the case with antimycin A. When chloramphenicol (2.0 mg/ml) was added to an 11-hr culture of wild type, cyanide-resistant respiration did appear (51 μliters of $O_2$ per hr per mg dry wt, or about one-third as much as for a 3-hr culture).

The detailed basis for the chloramphenicol effect has not yet been investigated. It is likely that, at the concentration used (2.0 mg/ml), chloramphenicol inhibits mitochondrial protein synthesis in vivo (Rifkin, Ph.D. Thesis, Rockefeller Univ., New York, N.Y., 1969). If that is the case, the fact that wild-type *Neurospora* can develop cyanide-resistant respiration in the presence of chloramphenicol indicates that mitochondrial protein synthesis is not involved.

TABLE 3. *Development of cyanide-resistant respiration by wild-type Neurospora in response to cyanide*[a]

| Cyanide concn (mM) | Maximal rate of respiration insensitive to 1 mM cyanide[b] |
|---|---|
| 0.09 | 59 |
| 0.23 | 47 |
| 0.50 | 21 |

[a] Respiration measurements were made at 3- to 4-hr intervals after the addition of cyanide to early exponential-phase wild-type cultures. At 0.09 mM cyanide, the cells resumed cyanide-sensitive respiration 5 hr after the addition of the inhibitor. Respiration measurements were terminated after an additional 5 hr. At 0.23 mM cyanide, resumption of cyanide-sensitive respiration occurred at 15 hr, and respiration measurements were terminated 7 hr later. At 0.50 mM cyanide, the cells did not resume cyanide-sensitive respiration during the experiment. Respiration measurements were terminated 17 hr after the addition of the inhibitor.

[b] Expressed as microliters of $O_2$ per hour per milligram (dry weight) of cells.

## DISCUSSION

Our results indicate that *poky* utilizes two oxidase systems, one sensitive to SHAM and the other sensitive to cyanide or antimycin A. The properties of these oxidases are consistent with the idea that they form a branched electron transport system (Fig. 12) similar to the one recently demonstrated for some higher plants (1, 5, 19, 24, 25, 26). A critical evaluation of this model, as it applies to *poky*, will be presented at a later time (Lambowitz et al., *manuscript in preparation*). The present discussion focuses on the properties of the two oxidase systems.

The SHAM-resistant system has a greater activity in *poky* $f^+$ than in *poky* $f^-$. It is probably a cytochrome system similar to that of wild type, since it can be inhibited by antimycin A and by 1 mM cyanide. This conclusion is compatible with reports that *poky* mitochondria contain a cyanide-sensitive cytochrome oxidase (Lambowitz et al., *submitted for publication*) and significant quantities of *b*- and *a*-type cytochromes (9, 18; Lambowitz et al., *submitted for publication*). The only complication arises from the fact that, in *poky* $f^+$, SHAM-resistant respiration is about 30

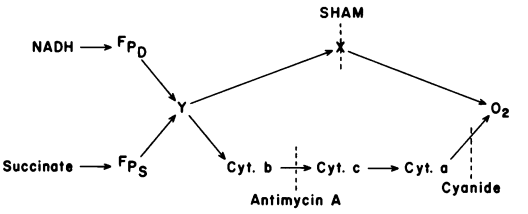


FIG. 12. *Branched electron transport system. Y designates the hypothetical branch point, and X designates the cyanide-insensitive oxidase, in accordance with Storey and Bahr (25). Inhibition sites are indicated by dashed lines.*

times less sensitive to antimycin A than is the wild-type cytochrome system. (A comparable experiment has not been performed on *poky* f⁻ because of the low level of SHAM-resistant respiration.) Although the decreased sensitivity could indicate an alteration of the cytochrome chain near the antimycin A-sensitive site, several other explanations are possible—for example, a modification of the cell membrane or intracellular environment which impeded the attack of the drug on the sensitive site.

The cyanide- and antimycin A-resistant system utilizes an oxidase (X; see Fig. 12) which is sensitive to SHAM. If one assumes a branched electron transport system, the data presented in this paper indicate that X contacts the respiratory chain on the substrate side of the antimycin A-sensitive site. More recent work with isolated mitochondria suggests that the point of contact lies between the dehydrogenases and the *b*-type cytochromes (Lambowitz et al., *submitted for publication*).

Wild-type *Neurospora* produces X when its cytochrome system is inhibited by antimycin A or cyanide, or when mitochondrial protein synthesis is inhibited by chloramphenicol. This fact suggests that cyanide-resistant respiration in *poky* is a secondary result of the *poky* mutation. Stimulation of cyanide-resistant respiration by antimycin A has also been reported in *Aspergillus oryzae* (10, 11) and *Euglena gracilis* (20). In both cases, cyanide resistance seems to be conferred by an alternate oxidase which contacts the electron transport chain on the substrate side of the cytochrome system, but it has not been reported whether the alternate oxidases in *Aspergillus* and *Euglena* are sensitive to hydroxamic acids.

The physiological role of cyanide-resistant respiration is not yet clear. In *Neurospora*, cells which use this pathway predominantly (*poky*, wild type grown in the presence of antimycin) have conspicuously slow growth rates, which seem to be related to the residual activity of the cytochrome system rather than to the level of the alternate oxidase (Fig. 8 and 9). Further work, with isolated mitochondria, will be required to show whether the cyanide-resistant pathway is capable of oxidative phosphorylation, or whether its role is limited to the oxidation of reducing equivalents.

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