SELECTION OF EXTRANUCLEAR MUTANTS OF *NEUROSPORA CRASSA*

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ABSTRACT

A procedure is described that produces primarily extranuclear mutants of *Neurospora crassa*. An analysis of five mutants, [*cni-3*], [*cni-4*], [*rsp-2*], [*rsp-3*], [*rsp-4*] is presented. All five mutants segregate in an extranuclear manner. They can be assorted into two classes based on their respiratory properties: (1) those with cyanide-insensitive respiration (*cni*); (2) those with slow respiration (*rsp*). All of the mutants are female sterile. The respiratory trait can be placed in different nuclear backgrounds by heterokaryotic transfer. The abnormal respiratory traits are observed in mitochondria isolated from the mutants and it is likely that the mutations are in mitochondrial DNA.

THE biogenesis of mitochondria is of considerable interest to investigators because of the many important biological functions carried out by these organelles. It is especially interesting to geneticists since both the nuclear and mitochondrial genomes contribute products that are required for mitochondrial assembly. It is now clear from a variety of studies that most mitochondrial proteins are coded for by nuclear genes, synthesized on cytoplasmic ribosomes and subsequently transported into the mitochondrion (SCHATZ and MASON 1974). Mitochondrial DNA has been shown to code for mitochondrial ribosomal RNA and mitochondrial transfer RNA but to date no polypeptide has been shown to be a gene product of mitochondrial DNA (MAHLER 1973).

A small number of polypeptides, possibly eight to twelve, are translated on mitochondrial ribosomes and integrated into the mitochondrial inner membrane (MICHEL and NEUPERT 1973; TZAGOLOFF 1973). These polypeptides have been shown to be associated with the cytochrome oxidase (SEBALD, MACHLEIDT and OTTO 1973), cytochrome *b* (WEISS and ZIGANKE 1974; WEISS, SCHAWB and WERNER (1975), and oligomycin sensitive ATPase complexes of the inner membrane (TZAGOLOFF 1971). While site of translation (cytoplasmic or mitochondrial ribosomes) cannot at present be correlated with genetic origin (nuclear or mitochondrial DNA), evidence has been presented to suggest that all polypeptides translated on mitochondrial ribosomes do arise from mitochondrial DNA (MAHLER and DAWIDOWICZ 1973). In addition to their possible function as components of the mitochondrial inner membrane, reports have been presented to suggest that mitochondrial gene products may act to regulate the expression of nuclear genes (BARATH and KUNTZEL 1972; EDWARDS, ROSENBERG and MARONEY, 1974, EDWARDS and ROSENBERG 1976).

In order to carry out a detailed analysis of the function of mitochondrial DNA, it would be desirable to have a method available that would primarily produce mutations in mitochondrial DNA. Extranuclear mutants of Neuospora that have been isolated have been widely studied (BERTRAND and PITTENGER 1972a; MITCHELL and MITCHELL 1952; JAGOW, WEISS and KLINGENBERG 1973) but selecting for mutants has been hampered by the lack of a suitable selective technique (BERTRAND and PITTENGER 1972a). A method that produces primarily extranuclear mutants of yeast has been reported by SHANNON *et al.* (1973). This method makes use of the observation that cycloheximide blocks nuclear DNA replication while allowing mitochondrial DNA replication to continue. Cells, can, therefore, be poisoned with cycloheximide and at the same time pulsed with a DNA base analog which becomes preferentially incorporated into mitochondrial DNA. SHANNON *et al.* (1973) have successfully used this method to produce extranuclear mutants of yeast that are resistant to oligomycin. We have applied this technique to conidia from *Neurospora crassa* and utilized a previously reported procedure for selecting respiratory deficient mutants (EDWARDS, KWIECINSKI and HORSTMANN, 1973). In this communication we present an anlysis of the first five mutants isolated by this procedure. All five have respiratory properties that are markedly different from the parent strain used in the mutation studies. All five proved to be extranuclear mutations.

MATERIALS AND METHODS

The following strains were obtained from the Fungal Genetic Stock Center (FGSC), Arcata, California: *in1–89601* (FGSC 497), *nic-1* (FGSC 763), *pan-1* (FGSC 5531), *leu-1* (FGSC 168), *alb-2* (FGSC 911). All of the strains were back-crossed until they were heterokaryon compatible. Heterokaryon compatibility between two auxotrophs was taken as the ability to grow in a normal manner on a growth tube of minimal medium. All of the strains used in the heterokaryon experiments were of mating type A.

Selection of mutants

Portions of the selection procedure have been published elsewhere (EDWARDS, KWIECINSKI and HORSTMANN, 1973). For the sake of clarity, however, the entire method is presented here.

i) *mutagenesis*—Conidia from the inositol-requiring strain *in1–89601* were germinated for four hours with shaking at 30° in Vogel's minimal medium (VOGEL 1964) supplemented with 2% sucrose and 50 μg/ml was added and incubation was continued for an additional 20 minutes. 2-amino purine (dissolved in absolute ethanol) was then added at a final concentration of 20 μg/ml and incubation was continued for 2 hours. Cells were then collected on a Millipore filter and washed twice with sterile water.

ii) *inositol-less death*—The washed cells were suspended in 10 ml of sterile water and added to 300 ml of acetate-minimal medium (EDWARDS, KWIECINSKI and HORSTMANN (1973) without inositol. The suspension was shaken at 30°, 200 rpm for 48 hours. At the end of the incubation, cells were collected on a Millipore filter, suspended in sterile water and plated on 50 plates of medium consisting of 1% sorbose, 0.05% glucose, 0.5% fructose, 2% agar, Vogel's salts and 50 μg of inositol per ml. The plates were incubated at 30° for 2–3 days.

iii) *tetrazolium overlay*—Colonies that grew on the plates were overlayed with a solution containing 0.1 *M* sodium phosphate pH 7.0, 1.5% agar and 0.5% 2,3,5-triphenyl-2H-tetrazolium chloride. The plates were then incubated at 39° for 3 hours. At the end of this time, the plates were scanned visually and colonies that failed to reduce tetrazolium (white) were isolated and transferred to slants for further analysis.

iv) *respiration measurements*—Respiration measurements were carried out at 30° on liquid-shaking cultures grown from the tetrazolium-negative isolates described above. Details of the measurements have been described (EDWARDS, KWIECINSKI and HORSTMANN, 1973).

v) *yield of mutants*—In a typical experiment, 500–1,000 or approximately 0.001% of the cells survive the inositol-less death. Of the survivors, approximately 10% (50–100) were tetrazolium negative. When the respiratory properties of these isolates were measured, usually 1–3 mutants were found.

Genetic Studies

Crosses were carried out on solid WESTERGAARD and MITCHELL's medium supplemented with 50 μg/ml inositol as described by DAVIS and DE SERRES (1970).

Heterokaryons were formed on slants of VOGEL's minimal medium by spotting conidia from two different auxotrophs. The conidial ratio used was approximately 50:50. Nuclear ratios were determined for each of the heterokaryons studied. These ratios were all approximately 50:50. Single auxotrophic colonies were then re-isolated by plating on minimal medium supplemented with the appropriate supplement. Concentrations of supplements used were: inositol, 50 μg/ml; L-leucine, 200 μg/ml; nicotinamide, 10 μg/ml. Colonies growing on the supplemented plates were isolated and tested for heterokaryosis by plating on minimal medium. True auxotrophs were then utilized for respiratory studies.

Studies with isolated mitochondria

Mitochondria were isolated from 24 h. cultures of the mutants by the method of LAMBOWITZ *et al.* (1972) with the exception that cells were treated with 20,000 units of β-glucuronidase (Sigma Type H-2) per gram wet weight instead of the snail gut enzyme, Glusulase. Respiration measurements of isolated mitochondria were made as described previously (EDWARDS, ROSENBERG and MARONEY 1974).

Spectral studies were made on succinate/anaerobic minus oxidized spectra using the extinction coefficients and wavelength pairs previously reported (LAMBOWITZ *et al.* 1972).

Miscellaneous procedures

Growth experiments were carried out at 26° as described previously (EDWARDS, KWIECINSKI and HORSTMANN 1973). Proteins were determined by the methol of LOWRY *et al.* (1951) using bovine serum albumin as a standard.

RESULTS

Respiratory properties of the mutants

Respiratory properties of mutants isolated by the procedure described in the METHODS section are shown in Table 1. Conidia from *in1–89601* were mutagenized by incubation with 2-amino purine in the presence of cycloheximide and then subjected to inositol-less death on acetate medium. Growth under these conditions requires mitochondrial function for energy production. Survivors of inositol-less death were then plated on medium containing glucose and fructose as the carbon source and the resultant colonies were overlayed with tetrazolium. Colonies that failed to reduce tetrazolium were selected and their respiratory properties were measured. Previous studies of this type (EDWARDS, KWIECINSKI and HORSTMANN (1973) had indicated that the respiratory properties of mutants obtained in this manner divide them into two distinct classes: a) Those with cyanide-insensitive respiration (*cni*); and b) those with slow respiration (*rsp*). Both classes of mutants were found in the present study. The *cni* mutants have high rates of respiration that are not significantly inhibited by the addition of

cyanide. This cyanide-insensitive respiration is inhibited by salicyl hydroxamic acid (SHAM) (LAMBOWITZ and SLAYMAN 1971; EDWARDS, KWIECINSKI and HORSTMANN (1973). The *rsp* mutants shown in Table 1 in all have respiratory rates that are less than 50% of the rate of the parent strain (*in1–89601*) from which they were isolated. This slow rate of respiration is sensitive to inhibition by cyanide. All of the *rsp* mutants reported here retain the capacity to produce the cyanide-insensitive respiratory pathway which can be induced in these cultures by treatment with chloramphenicol (EDWARDS, ROSENBERG and MARONEY 1974). This pathway is not expressed in these mutants, however, under normal conditions. Also shown in Table 1 is the respiratory properties of an isolate we have called *nmw-1* (non-mutant white). This is presented as an example of a colony that survived the inositol-less death and failed to reduce tetrazolium but had wild-type respiratory properties.

Genetic Studies

Reciprocal crosses were carried out between the mutants (inositol-requiring) and a leucine auxotroph (*leu-1*). When the mutants were used as the proto-perithecial parent, they all proved to be female sterile and produced few, if any, viable spores. When the mutants were used as the conidial parent, however, viable spores were obtained. A random spore analysis was carried out on a minimum of 50 spores from each of these crosses. In all cases, none of the spores had the respiratory properties of the respiratory-deficient parent. All of the spores had high levels of respiration that was sensitive to cyanide. The nuclear markers, inositol and leucine, segregated 1:1. The germination rates in these studies ranged from 90–93% so it is not likely that a class spores was missed because it failed to germinate. In addition to these experiments, asci were also dissected from crosses of the respiratory mutants to *alb-2*, an albino mutant with cyanide-sensitive respiration similar to that found in wild-type cells. Mutant *cni-4* was not included in these studies as germination and growth of ascospores is poor even when the mutant is used as the conidial parent. Five complete asci were

TABLE 1

Respiration rates of respiratory-deficient mutants

Strain	Respiration rate (μl O_2/hr/mg)	+KCN	+KCN + SHAM
cni-3	90.40 ± 1.07	64.60 ± 1.0	3.09 ± 0.58
cni-4	94.68 ± 2.00	75.20 ± 0.80	4.66 ± 0.87
rsp-2	22.37 ± 1.34	1.00 ± 0.44	—
rsp-3	42.10 ± 1.6	2.41 ± 0.51	—
rsp-4	43.11 ± 2.21	2.32 ± 0.83	—
in1–89601	102.74 ± 2.71	4.89 ± 1.10	—
nmw-1	116.42	7.72	—

Measurements were made on cultures grown for 24 hours at 30° in Vogel's minimal medium supplemented with 200 μg/ml inositol. Values are the mean ± 1.0 standard error of the mean for at least five determinations. The final KCN concentration used was 1.0 millimolar. The final SHAM concentration was 120 μg/ml. SHAM inhibition was only measured on the *cni* strains.

isolated from each of the crosses. In all cases the spores had the respiratory properties of the *alb-2* parent. Respiration was high (~100 μl O_2/hr/mg) and was inhibited by cyanide. The *alb-2* marker and its wild-type allele segregated 4:4 in each of the asci examined. In no case among the 160 ascospores that we examined did we find a spore with the respiratory properties of the mutant used in the cross.

Formation of Heterokaryons

These results indicate that the respiratory deficiency of these mutants segregates in an extranuclear manner, but are not entirely satisfactory since the mutants are all female sterile. In order to overcome this difficulty, experiments were carried out to determine whether the respiratory-deficient trait could be transferred through a heterokaryon. Heterokaryons were formed between one of the mutants (inositol-requiring) and either the *leu-1*, *nic-1* or *pan-1* auxotroph. In all cases that we studied, the formation of a heterokaryon was taken as the ability to grow in a normal manner on a growth tube of minimal medium. None of the individual components of the heterokaryons would grow on minimal medium. The auxotroph was then re-isolated and its respiratory properties were measured. When the respiration rates of different isolates of the auxotroph were measured, a broad range of respiratory properties was seen. The respiration ranged from high to low and from cyanide-sensitive to cyanide-insensitive depending on which mutant was being studied. When the inositol auxtroph was reisolated, a broad range of respiratory properties was again seen. In all cases, we were able to find an isolate of the auxotroph that had the same respiratory properties as the mutant being studied. Examples of these isolates are shown in Table 2. In all cases that we tested the respiratory deficient trait could be transferred to a variety of nuclear backgrounds by heterokaryotic transfer. This result demonstrates that the respiratory properties of these five mutants can be transmitted in an extra-nuclear manner. In subsequent studies (see below) we found that we could readily select isolates of the auxotrophs that had the respiratory-deficient trait by using the tetrazolium overlay method (EDWARDS, KWIECINSKI and HORSTMANN 1973).

Attempts were also made to use the heterokaryons that we formed as the protoperithecial parents in crosses to both *nic-1* and *alb-2*. Under these conditions asci with viable ascospores could be isolated, but in all cases the respiratory properties of the progeny were those of the respiratory-competent component of the heterokaryon. Progeny with the *in1* marker were present but these all had high respiration rates that were sensitive to cyanide. We found no cases where mutant respiratory properties were transmitted in these crosses.

Validity of the tetrazolium overlay method

The heterokaryotic transfer experiments also provided a method for testing the validity of the tetrazolium overlay method used in the selection procedure. It was deemed necessary to test this method since other genes have been described which affect tetrazolium reduction and are not related to respiratory deficiency (GILLIE 1970).

TABLE 2

Respiration rates of mutants isolated by heterokaryotic transfer

Respiratory mutant	Respiration rate (μl O_2/hr/mg)		
	nic-1	*pan-1*	*leu-1*
cni-3	92.10 ± 1.89	—	93.11 ± 0.90
+KCN	76.40 ± 0.74	—	76.83 ± 2.13
+KCN + SHAM	7.61 ± 0.38	—	6.63 ± 0.94
cni-4	96.30 ± 2.13	—	103.18 ± 2.13
+KCN	78.90 ± 1.15	—	77.85 ± 1.74
+KCN + SHAM	0.32 ± 0.09	—	8.68 ± 0.57
rsp-2	41.33 ± 0.03	55.76 ± 1.95	34.80 ± 2.01
+KCN	3.02 ± 0.39	1.72 ± 0.07	0.0
rsp-3	38.76 ± 2.02	35.08 ± 2.39	28.59 ± 0.77
+KCN	4.82 ± 1.10	1.52 ± 0.20	0.0
rsp-4	33.54 ± 1.75	46.70 ± 20.06	45.20 ± 1.31
+KCN	4.21 ± 0.26	3.05 ± 0.61	3.60 ± 0.90
nic-1	96.70 ± 2.03	—	—
+KCN	2.80 ± 0.90	—	—
pan-1	—	107.20 ± 2.16	—
+KCN	—	5.90 ± 1.09	—
leu-1	—	—	110.70 ± 2.09
+KCN	—	—	3.42 ± 0.89

Strains were constructed by formation of heterokaryons on minimal medium between one of the respiration mutants (inositol-requiring) and one of the auxotrophs shown in the table. The auxotroph was then reisolated by plating on minimal medium containing the appropriate supplement and checked for heterokaryosis by plating on minimal medium. Individual reisolates of the auxotroph were then screened for their respiratory properties. Respiration rates were measured at 30° in Vogel's minimal medium containing 2% sucrose and the appropriate supplement (nicotinamide, pantothenic acid or leucine). Rates are reported as the mean ± 1.0 standard error of the mean for at least five determinations. Respiration rates of the mutants in the inositol nuclear background are in Table 1. The final concentration of KCN was 1.0 millimolar. The final SHAM concentration was 120 μg/ml.

In order to test this method directly, a heterokaryon was formed between mutant *cni-3* and *leu-1*. The nuclear ratio (DAVIS and DE SERRES 1970) of this heterokaryon was 46% *inl* and 54% *leu*. Conidia from the heterokaryon were plated on appropriate media in order to determine the segregation ratio of nuclear markers. Of 460 colonies that were screened, 32.6% were *inl*, 37.0% were *leu* and 30.4% were heterokaryotic. When conidia from isolates of any of these nuclear types were plated and overlayed with tetrazolium, a mixture of tetrazolium reducing properties was seen. Colonies that reduced tetrazolium (red), failed to reduce tetrazolium (white) and of an intermediate nature (pink) could be observed.

A detailed analysis of the tetrazolium-reducing properties of conidia from a leucine-requiring isolate from the heterokaryon was then carried out. Conidia were plated on leucine-containing plates and overlayed with tetrazolium after

the colonies had grown. Approximately 1600 colonies wer screened and of those 40% were white, 30% were red and 30% were pink. Individual isolates of white, red, and pink colonies were taken and the respiratory properties of cultures derived from these were measured. Eleven white colonies were analyzed and of these two had high levels of cyanide-sensitive respiration and nine had cyanide-insensitive respiration. The percentage of cyanide-insensitive respiration in these cultures ranged from 18.4 to 100% insensitive. In all cases, the cyanide-insensitive respiration was inhibited by salicyl hydroxamic acid. Twenty-six red colonies were analyzed in the same manner and all of these had high levels of cyanide-sensitive respiration. The range of cyanide-insensitive respiration was from 0 to 8.2%. Sixteen pink colonies were also analyzed and the range of cyanide-insensitive respiration was from 0 to 16.5%. Respiration rates for cultures from all of the tetrazolium negative colonies and representative examples of the red and pink colonies are shown in Table 3. We note that by using the tetrazolium overlay method we were able to isolate *cni-3* colonies that had a higher percent of cyanide-insensitive respiration than the original isolate (compare Table 3 with Table 1). There are at least two possible explanations for this. The first is that the original *cni-3* isolated is still a heteroplasmon and contains some respiratory-competent mitochondria. These mitochondria can then segregate

TABLE 3

Respiration rates of isolates from tetrazolium overlay of colonies from a heterokaryon

Isolate		Respiration rate (μl O_2/hr/mg)	
		+KCN	+KCN + SHAM
W-1	120.85	117.62	0.89
W-2	99.00	93.35	2.01
W-3	81.30	80.62	1.19
W-4	116.53	116.39	0.00
W-5	121.47	116.14	1.76
W-6	83.97	83.83	0.72
W-7	109.02	109.49	0.24
W-8	115.0	96.20	1.61
W-9	97.75	75.80	0.0
W-10	97.86	34.57	0.72
W-11	84.48	15.71	0.0
P-1	97.76	0	0
P-2	95.76	6.69	0
P-3	86.70	22.66	1.3
R-1	93.11	0	0
R-2	110.93	4.66	0
R-3	99.79	8.15	0.34

A heterokaryon was formed between *cni-3* and *leu-1*. A leucine auxotroph was reisolated from the heterokaryon and conidia from this isolate were plated on leucine-containing plates. The resultant colonies were overlayed with tetrazolium and red, white and pink colonies were isolated. Liquid shaking cultures of these isolates were grown for 24 hours at 30° and respiratory properties measured. Nomenclature used: W, white; R, red; P, pink. The red and pink isolates represent the highest, lowest and median respiration observed. Concentrations of KCN and SHAM were the same as in Table 2.

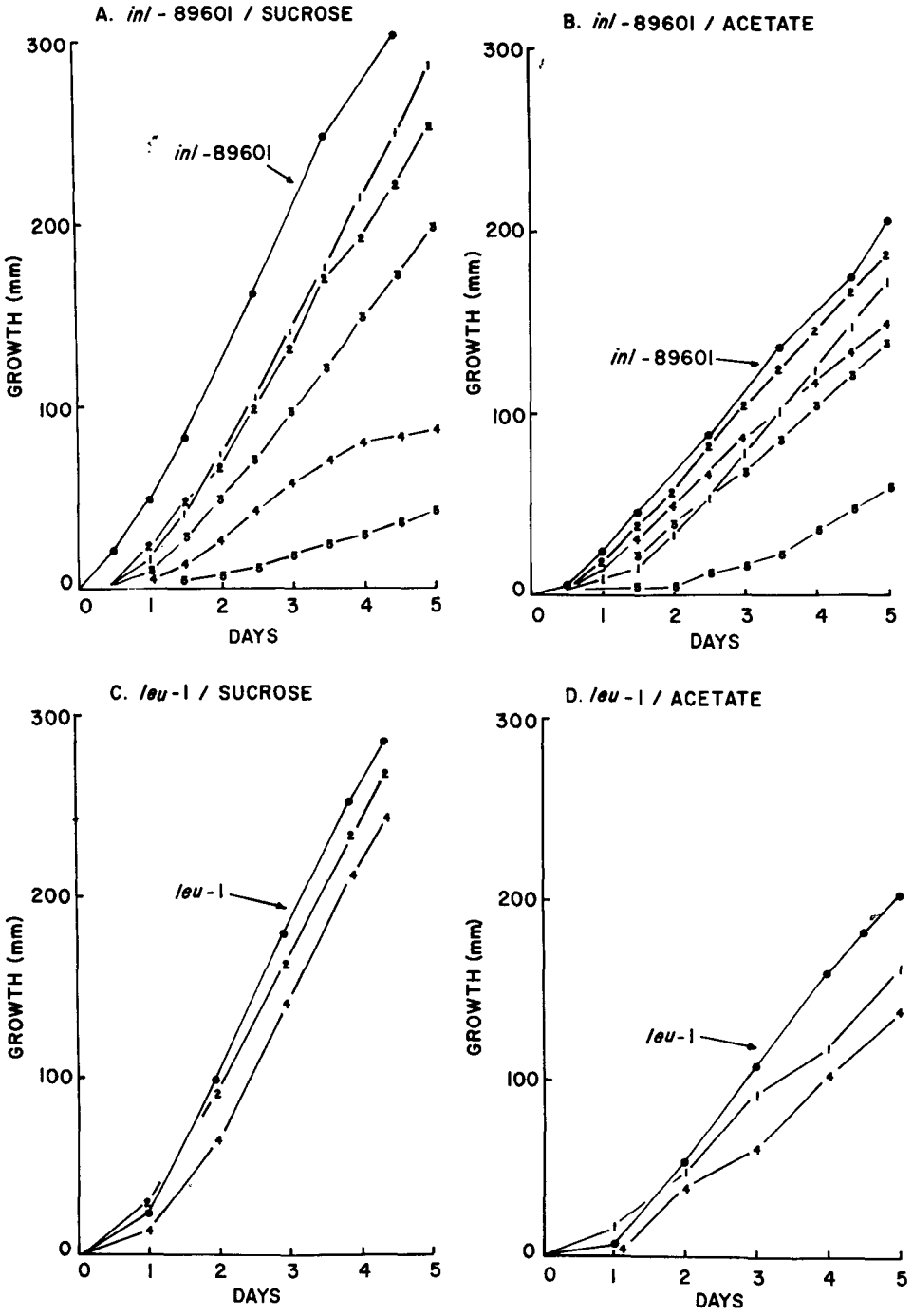

FIGURE 1.—Growth rates of parent and respiratory-deficient mutant strains on growth tubes. Studies were carried out on mutants in the *inl–89601* and *leu-1* nuclear backgrounds. (A): growth of mutants in the *inl* background on sucrose medium. Numbers used to represent

randomly into conidia. The second possibility is that the amount of cyanide-insensitive respiration may vary with the age of culture. This phenomenon has been clearly demonstrated for the *cni-1* mutant of Neurospora (KLEIN, EDWARDS and WERNER 1975). These arguments also apply to the variable rates of cyanide-insensitive respiration of the tetrazolium-negative colonies shown in Table 3. While cultures for the present study were all grown for 24 hours at 30°, differences in inoculum size or in conidial viability may have some effect on the results.

Conidia from all of these isolates were also replated and again overlayed with tetrazolium in order to determine whether the tetrazolium-reducing properties were stable. When the white isolates were tested in this manner they gave rise to both white and pink colonies with the range of pink colonies varying from 0–25%. These isolates that had 100% cyanide-insensitive respiration by the oxygen-electrode analysis yielded only white colonies, while those with lesser amounts of cyanide-insensitive respiration yielded a mixture of white and pink colonies. The pink colonies on these plates were readily distinguishable from the red colonies of tetrazolium-positive strains. When the red isolates were tested, they gave mixtures of red and pink colonies with the percentage of pink colonies in varying proportions. No further analysis was carried out on these isolates.

Similar experiments were also carried out on heterokaryons formed between the respiratory mutants and the *nic* and *pan* auxotrophs. In all cases it was possible to isolate a tetrazolium-negative auxotroph that had the respiratory properties of the original mutant. The results with the pantothenate auxotroph were not as clearcut as with the other auxotrophs. In this case, isolates that had mutant properties were slightly pinkish in color. They were easily distinguishable from respiratory component colonies but were, nevertheless, not colorless. The reason for this slight anomaly is not known. Control experiments with the *in1*, *leu*, *nic* and *pan* strains all gave colonies that were uniformly red in color. The respiratory mutants all gave uniformly white colonies when overlayed with tetrazolium.

Growth Studies

The growth of mutant and parent strains on growth tubes are shown in Figure 1. All of the mutants grow at a slower rate than the parent strain using either sucrose or acetate as a carbon source. When the mutant phenotype is in an inositol nuclear background, there is a broad differential between the growth of some of the mutants and the parent strain (Figure 1A, B). When the mutants

various mutants are 1–1, [*rsp-2*]; 2–2, [*rsp-3*]; 3–3, [*rsp-4*]; 4–4, [*cni*-3]; 5–5, [*cni-3*]. (B): Growth of the same mutants on acetate medium. Numbering is the same as in A. (C): Growth of mutants in the *leu* nuclear background on sucrose medium. Only curves for [*rsp-3*] (2–2) and [*cni-4*] (4–4) are shown. Growth curves of all other mutants fell within the area bounded by the curves for [*rsp-3*] and [*cni-4*]. (D): Growth of the *leu* mutants on acetate medium. Only the curves for [*rsp-2*] (1–1) and [*cni-4*] (4–4) are given. Growth curves for all other mutants fell within the area bounded by the curves for [*rsp-2*] and [*cni-4*]. All experiments were carried out at 26°.

are transferred heterokaryotically to a leucine nuclear background, this differential is greatly reduced (Figure 1C, D). This suggests that the slow growth seen in some of the mutants in the inositol background is not due entirely to the extrachromosomal mutation but is due in part to the inositol mutation. This effect may be due to the involvement of inositol in the synthesis of cellular membranes. It is also possible, however, that the cytoplasmic determinants are not fully and efficiently transferred in these experiments and that the incomplete transfer results in the altered growth rates observed. We have not been able to differentiate between these possibilities.

Properties of isolated mitochondria

In order to demonstrate directly that the abnormal respiratory properties exhibited by the mutants were due to defective mitochondrial respiration, studies were carried out with isolated mitochondria. Respiration rates of mitochondria isolated from mutants which had been transferred to the leucine nuclear background are shown in Table 4. Mutants *cni-3* and *cni-4* retain high levels of cyanide-insensitive respiration using either succinate or NADH as a substrate. The cyanide-insensitive respiration present is inhibited by salicyl hydroxamic acid. We note, however, that the cyanide-insensitive pathway is labile and is rapidly lost soon after the mitochondria are isolated (EDWARDS, ROSENBERG and MARONEY, 1974). It is not recovered in either the low speed pellet or post-mitochondrial supernatant fractions obtained during the isolation of the mitochondria.

Mitochondria from the three *rsp* mutants all have reduced rates of respiration with either succinate or NADH as a substrate. This is consistent with the reduced respiratory rates seen with whole mycelium of these mutants. These studies demonstrate that the abnormal respiratory properties of the mutants are due to abnormal mitochondrial respiration. All of the mutants that we have studied exhibit respiratory control (Estabrook 1967) when ADP is added to mitochondria respiring in state 4. It is therefore unlikely that the respiratory properties observed are due to damage to the mitochondria during isolation.

TABLE 4

Respiration rates of isolated mitochondria

Substrate	Respiration rate (μAtoms O/min./mg)					
	cni-3	*cni-4*	*rsp-2*	*rsp-3*	*rsp-4*	*leu-1*
Succinate	.316 ± .005	.379 ± .003	.144 ± .034	.149 ± .019	.162 ± .007	.484 ± .002
+KCN	.114 ± .080	.195 ± .002	0.0	0.0	0.0	.002 ± .001
NADH	.359 ± .002	.550 ± .002	.187 ± .019	.189 ± .002	.241 ± .010	.524 ± .004
+KCN	.116 ± .001	.243 ± .003	0.0	0.0	0.0	0.0

Measurements were carried out at 30° on mitochondria isolated from 24 h cultures. Values are reported as the mean ± 1.0 standard error of the mean for at least 5 determinations. The concentration of NADH used was 0.5 millimolar. The concentration of succinate was 10 millimolar. The first concentration of KCN was 1.0 millimolar.

Spectral studies

Since many extrachromosomal mutants of Neurospora have abnormal concentrations of cytochromes (BERTRAND and PITTENGER 1972a; JAGOW, WEISS and KLINGENBERG 1973), it was of interest to determine the cytochrome content of the five mutants presented in this study. We were surprised to find that none of the mutants reported here have grossly abnormal cytochrome content as determined from room temperature spectra. All five mutants contain cytochromes *c*, *b* and aa_3 in concentrations that are not significantly different from those of the *in1–89601* parent strain. We have not attempted any low-temperature spectral studies with these mutants which would reveal minor differences in cytochrome content.

DISCUSSION

The method that we describe here appears to us to be a sound one for isolating extranuclear mutants of Neurospora. We have found that if the procedure is followed exactly, the yield of extranuclear mutants is high and may be 100%. While no single step in the procedure provides unambiguous selection, the combination of inositol-less death, tetrazolium overlay and measurement of respiration provides a mechanism by which respiratory mutants can be easily obtained (EDWARDS, KWIECINSKI and HARTMANN 1973). The addition of 2-amino purine to cycloheximide-poisoned cells appears to be as effective in Neurospora as it has been shown to be in yeast for generating extranuclear mutants (SHANNON *et al.* 1973). Since the method selects for mutants with defective mitochondrial respiration, and we have demonstrated that the altered respiratory properties are indeed mitochondrial and are non-nuclear in transmission, it seems likely that the mutations are in mitochondrial DNA. This method, then, appears to be a powerful tool for a study of the mitochondrial genome. The number of mitochondrial gene products must be relatively small due to the limited amount of mitochondrial DNA that is present, and a relatively small number of mutant classes should be obtained.

We are aware that tetrazolium overlay procedures have been used for some time in a number of laboratories in attempts to select for respiratory-deficient mutants. The results of such studies have not been conclusive. We believe that the data presented here provide experimental evidence that the tetrazolium overlay method can be used effectively in selecting for respiratory mutants. Our studies on the segregation of respiratory markers from heterokaryons having both mutant and wild-type mitochondria indicate that the failure to reduce the dye can be correlated with the respiratory-deficient phenotype. Isolates can be obtained by sub-culturing that either have mutant properties and are completely tetrazolium-negative or have wild-type properties and are completely tetrazolium-positive. These observations indicate that the variation in dye color is a reliable indicator of respiratory properties. The results are not entirely unambiguous, however, and we have found some isolates that are tetrazolium-negative but with wild-type respiratory properties (Table 1). Whether these are related to previously described genes (GILLIE, 1970) or to new types of mutations is not known. We

have, however, never observed the converse of this phenomenon, i.e., a tetrazolium-positive isolate with mutant respiratory properties.

A critical factor in these studies is the time course of the tetrazolium overlay. We have carried out preliminary studies on the *in vitro* reduction of tetrazolium by isolated mitochondria. We have found that mitochondria from *cni-3* reduce tetrazolium *in vitro* at approximately 50% of the rate observed for wild-type mitochondria. Blocking the cytochrome chain of *cni-3* with cyanide results in a tetrazolium-reduction rate of approximately 25% of the rate for wild-type mitochondria. This reduction of tetrazolium can occur due to the cyanide-insensitivity pathway. These studies indicate that the time of incubation in the presence of tetrazolium is critical to the success of the method. Incubation for too long a period of time will result in a reduction of tetrazolium by the reduced capacity of a mutant cytochrome chain or by the cynanide-insensitive pathway. We have found that the conditions used in the selection technique we have described (39°, 3 hours) are sufficient to distinguish between normal and mutant phenotypes.

It seems likely, therefore, that the transfer of tetrazolium-reducing properties in the heterokaryon experiments reflects the transfer of the respiratory mutant phenotype from the original mutant strain. Since the properties of the isolates are always the same as those of the original mutant (i.e. *cni* or *rsp*) it is not likely that the mutant properties observed in the isolates are due to new mutations.

The studies that we have carried out to date with heterokaryons formed between mutant and respiratory competent strains have not yielded information as to the dominance relationships of these mutants with the exception of some preliminary evidence concerning *cni-3*. Studies of the pink colonies arising from the heterokaryon between *cni-3* and *leu-1* (Table 3) indicate that it is recessive. Studies that we have published elsewhere, however, give an indication of the difficulties that are encountered in attempting to determine dominance relationships with these mutants (EDWARDS and ROSENBERG 1976). In these studies a balanced heterokaryon was formed between *cni-3* and *leu-1*. When the respiratory properties of the heterokaryon were measured the respiration was initially cyanide-sensitive. When the respiration of the heterokaryon was measured after 30–40 mass doublings, however, the respiration was cyanide-insensitive. These studies show that it will be difficult to determine the true dominance relationships as long as there may be a changing ratio of mutant and wild-type determinants.

Preliminary studies on growth tubes also indicate that heterokaryons between any *cni* and *rsp* mutant grow at a faster rate than either mutant alone. All of these studies indicate that it may be possible to classify mitochondrial genes by complementation analysis. Such an analysis of mitochondrial mutants has been presented for Neurospora (BERTRAND and PITTENGER 1972a, 1972b; PITTENGER 1956) and also for yeast (CLARK-WALKER and MIKLOS 1975). It should be pointed out that in the Neurospora studies by BERTRAND and PITTENGER (1972b) "complementing mutants" are mutants that give accelerated growth in pairwise combinations but retain abnormal cytochrome content. Recent studies (EDWARDS, ROSENBERG and MARONEY 1974; KLEIN, EDWARDS and WERNER 1975; EDWARDS and ROSENBERG 1976) have shown that regulatory interactions occur between the

mitochondrial and nuclear genomes. In view of these studies, and of the abnormal cytochrome spectra obtained in previous "complementation" studies, we feel that caution must be exercised in interpreting such results and that a great deal of investigation must yet be done before true complementation can be assessed.

The mutants that we describe here all differ in their properties from the other extranuclear mutants that have been studied (BERTRAND and PITTENGER 1972a). All of the previously reported mutants have abnormal cytochrome content while the mutants we report all have normal cytochrome content. We note that all of the previously reported mutants were selected for a readily detectable growth deficiency. The mutants we report grow more slowly than the parent strain but the grow deficiencies are not as extreme as those of the previously reported mutants, especially if they are not in an inositol nuclear background. The mutants that we describe may have been discarded as marginal in previous studies. It should also be pointed out that the mutants in this study are simply the first five isolates from the procedure we describe and that the purpose of this paper is only to provide the details of the method and demonstrate its workability. For these reasons we leave such questions as complementation analysis and the transmission of female sterility by the mutants for subsequent studies.

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