CHARACTERIZATION OF SEVERAL MITOCHONDRIAL VARIANTS
OF NATURAL ISOLATES OF NEUROSPORA INTERMEDIA

by

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B.Sc., The University of Vermont, 1978

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We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
April 1981

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Date April 24, 1981
ABSTRACT

A survey of natural isolates of *N. intermedia* revealed five variants with respect to growth phenotype. These variants showed stop-start behavior in growth tubes, sometimes never attaining the full tube length. These strains are mainly female sterile. The stop-start phenotype was not transmitted via the male parent in any cross. In two crosses, maternal transmission was demonstrated. One strain was investigated for the presence of virus-like particles, but none were found. Cytochrome spectra show a deficiency of cytochromes \( a \) and/or \( b \) relative to the amount of \( c \). In respiration studies, the one strain tested proved to be cyanide resistant and salicyl hydroxamic acid sensitive. These characteristics are also found in extranuclear mutants of *N. crassa*. However, the analogy no longer holds at the mitochondrial ribosome level. An analysis of four of the strains disclosed that three are large subunit deficient. Only small subunit deficient strains have been found among the cytoplasmic mutants of *N. crassa*.

Restriction enzyme analysis was also carried out on the variants. It was found that they possess additional mitochondrial DNA compared to normal *N. intermedia*. One Eco RI fragment of M.W. \( 2.4 \times 10^6 \) was found to be common to all variants tested, but is not found in the normal strains. There were other DNA differences which were unique to each abnormal strain. These findings suggest that the basis for the abnormal phenotypes is some heritable factor associated with the mitochondria.
Dedicated to the memory of my father. His enthusiasm for science and devotion to his work have been a true inspiration.
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SECTION I

Genetic Analysis
INTRODUCTION

In 1901 C. Correns and E. Baur (Correns, 1909; Baur, 1909) noted non-Mendelian patterns of inheritance for a factor influencing chloroplast development in some strains of flowering plants. Working with *Mirabilis*, Correns found maternal inheritance governed the presence of mutant white sectors of leaf tissue. Baur also found non-Mendelian ratios for the same trait in the genus *Pelargonium*. While they attributed the phenomenon to extrachromosomal heredity, little interest was aroused in the scientific community since work on chromosomal genetics was progressing so rapidly (Sager, 1972).

However, as more cases of cytoplasmic genes were discovered, their significance was soon recognized. A vast array of organisms was shown to possess extranuclear genes. These ranged from unicellular algae (*Chlamydomonas*, Sager, 1954) to complex higher plants (such as *Triticum*, Briggle, 1966). An example of the practical importance of extranuclear inheritance was found in *Zea mays* (Rhoades, 1933). A cytoplasmic gene causes male sterility and is consequently used in commercial corn hybridization to eliminate the cost of manual detassling. Also, in man, two congenital abnormalities, anencephaly and spina bifida, are suspected to be the result of cytoplasmic genes (Nance, 1969). Another case of extrachromosomal genes which is of human interest is seen in mosquitoes (Lavens, 1967). These insects carry a cytoplasmic incompatibility between different populations. When incompatible males are introduced the females will randomly mate with them reducing the number of the total population. This procedure was experimentally carried out in Burma when a mosquito population was abolished in a two
month period.

Further examples of cytoplasmic inheritance are continuously being discovered. Yet, little is known about these organelle genes: how they interact with nuclear genes, their importance in development of the organism and their evolution. Therefore, research in this area is becoming widespread.

The most studied organisms are the lower eukaryotes. One, the green alga *Chlamydomonas*, shows maternal inheritance for resistance to certain antibiotics, as well as temperature sensitivity, slow growth and photosynthetic mutations (Sager, 1972). Sager (1954) found that both parents contribute their cytoplasmic genes to the progeny. However, the DNA from the two parents are treated differently in the zygote so that its phenotype resembles the female parent (Sager and Lane, 1969).

Another well studied organism is *Saccharomyces cerevisiae*. A class of mutants of this yeast, referred to as cytoplasmic "petites" (Ephrussi and Hottinguer, 1951), shows non-Mendelian inheritance. These mutants are characterized by a small colony size and a deficiency in cytochrome activity. Some petites have also been shown to retain only about 0.1% of their mitochondrial DNA and others have an altered base ratio so that the G + C content is only 18% (Borst and Grivell, 1978). In the past decade, the yeast mitochondrial genome has been extensively mapped and much has been learned regarding which genes are located in the mitochondria and which are nuclear genes (Borst and Grivell, 1978). The genetic and physical map of yeast mtDNA is given in Figure 1.

The fungi, *Podospora* and *Aspergillus* also show cytoplasmic inheritance. The phenomenon of senescence in *Podospora* (Tudzynski and
Figure 1. Genetic and Physical Map of Yeast mt DNA.

The inner ring shows the location of various markers (i.e. erythromycin resistance). The black bars in the inner ring represent insertions present in the mt DNA of *S. cerevisiae* and absent in *S. carlsbergensis*. The outer ring gives the positions of recognition sites for endonucleases Hind II + III and Eco RI and the position of 4S RNA genes. The open circles are tRNA\textsubscript{met} genes. The approximate positions of other transcripts are given outside the outer ring, the bars indicating uncertainty in the exact positions. The open part of the 21S rRNA represents the intervening sequence. Sal and Pst indicate the single recognition sites for restriction endonuclease Sal I and Pst I, respectively. The figure and legend are taken from Borst and Grivell, 1978.
Esser, 1979), and sexual differentiation in *Aspergillus* (Mahoney and Wilkie, 1958), are controlled by extranuclear genes. The fungus *Neurospora crassa* has been the subject of much research. This species is an ideal genetic tool. The ease with which it is cultured and manipulated renders it invaluable. The first case of unusual growth patterns in *Neurospora* was found by Mitchell and Mitchell (1952). Their characterization of the strain [poky] (later referred to as [mi-1] by Mitchell et al., 1953) showed a greatly reduced growth rate with respect to normal strains (Figure 2). Utilizing genetic analysis they showed that this growth habit could only be inherited through the maternal parent (with rare exceptions given in the Discussion). They proposed a cytoplasmic factor as the cause, since the female presumably contributes the bulk of the cytoplasm in a cross. It has also been found that [poky] lacks cytochromes a and b and has an excess of c (Haskins et al., 1954). This fact, along with other mitochondrial abnormalities to be discussed in Section II, indicates that the unusual growth phenotype of [poky] is associated with some heritable factor within the mitochondria.

Subsequently, several other maternally inherited growth mutants have been found in *N. crassa*: maternally inherited-3 ([mi-3]), which shows slow growth and abnormal cytochromes (Mitchell et al., 1953); slow growth mutants ([SG]), these also show maternal inheritance, slow growth, and abnormal cytochromes (Srb, 1958); abnormals ([abn]), these strains show slow and abnormal growth, they are female sterile, so that cytoplasmic inheritance is indicated by non-Mendelian ratios and transmission of the irregular growth through heterokaryons; the [abn]'s also have a defective cytochrome system (Garnjobst et al., 1965);
Figure 2. Growth Curves of Poky vs. Not Poky.

Taken from Mitchell and Mitchell, 1952.
stoppers ([stp]) are characterized by irregular growth and abnormal cytochromespectra, they, too are female sterile (McDougall and Pittenger, 1966); and extranuclear mutants ([exn]), these have a pronounced "lag" phase (very slow growth at first, followed by much faster growth), an abnormal cytochrome system, and are female fertile (Bertrand and Pittenger, 1972).

All of the N. crassa growth mutants mentioned are laboratory derived strains. They either arose in laboratory stocks or were induced through mutation. Consequently, little is known regarding the adaptive significance, if any, of the unusual growth rates. If this behavior could be shown to exist in nature, it would provide an opportunity to explore its role, and, perhaps to understand how such an extraordinary growth habit could survive and compete in nature. Genetic and biochemical work on natural isolates possessing abnormal growth may add insight to the relationship between nuclear and cytoplasmic genes. Thus, it was indeed of interest when a population study, undertaken to investigate variation in growth rates among natural populations (carried out by Dr. A.J.F. Griffiths), of N. intermedia isolates, disclosed two with a stop-start growth pattern. The strains used for Griffiths' study were collected by D.D. Perkins from locations around the world. No abnormalities were noted when the strains were first isolated by Perkins (the strains are listed in Materials and Methods). Once these two exceptional isolates were recognized, a search was undertaken for additional strains with a similar growth phenotype. The strains used for this were also collected by Perkins and were from Kauai, Hawaii.

The research described in this thesis includes the identification of several strains among these natural isolates showing abnormal growth
rates. The objectives were not only to discover these strains, but to characterize them genetically and biochemically. If these strains could be shown to possess mitochondrial abnormalities, as do the \emph{N. orassa} strains, they would provide a new source of mutants with which to probe the mitochondrial genome. Also, information would be gained as to the ecological role of such strains and the mechanism of the mutation.

The first question which arises is whether or not the growth habit of the \emph{N. intermedia} isolates shows cytoplasmic inheritance. Several methods can be used in \emph{Neurospora} to find the mode of inheritance of this type of trait. Two of these are: 1) reciprocal crosses and, 2) heterokaryon testing. In reciprocal crosses, only the female parent should pass on its phenotype, if the trait is extranuclear. Thus, when an abnormal isolate is used as the female, the progeny should show the abnormal phenotype. When the normal strain is used as the female, the progeny should be normal.

It has been found in \emph{N. orassa} that the anomalous growth habit can be passed through a heterokaryon (Garnjobst \textit{et al.}, 1965). A heterokaryon consists of genetically distinct nuclei within a common cytoplasm. Heterokaryons are formed frequently in fungi and arise through hyphal anastomosis. A heterokaryon can be forced to occur if two compatible strains possessing different auxotrophic markers are inoculated onto minimal media together. Both cultures initiate limited growth. The hyphae fuse and the nuclei migrate through the cytoplasm enabling the culture to continue growing.

If however, the strains are incompatible, there is anastomosis, but the single fusion cell is blocked off from the rest of the hyphae
Nuclei can not migrate so no heterokaryon results. Both strains will cease growth due to the lack of a nutrient, the one required by the auxotrophic marker.

Heterokaryon compatibility in the species *N. crassa* requires that the two strains be of the same mating type. In other words, two strains which will form a sexual cross will not produce vegetative heterokaryons. However, not only must they be of like mating type, but they must also be homozygous at three other known loci: C, D, E (Garnjobst, 1953; Mylyk, 1975). An example of compatible strains would be two A mating type cultures both carrying the three recessive alleles: c, d, and e. Only when all these requirements have been met are the strains compatible and a successful heterokaryon formed. The conidia which are produced by a heterokaryon will consist of some which contain the nucleus of one strain with the cytoplasmic factors of the other (Figure 3).

Thus, a heterokaryon test can be used to show a cytoplasmic factor as the cause of a trait. When a normally growing strain and an abnormal growth phenotype strain are forced into a heterokaryon, some resulting conidia should possess the cytoplasm from the abnormal strain and nuclear markers of the normal strain.

The *N. crassa* cytoplasmic growth mutants which have been shown to pass their phenotype through a heterokaryon in this way are: [abn-1] and [abn-2] (Garnjobst et al., 1965), [mi-1] and [mi-4] (Pittenger, 1956), and [exn-1] and [exn-4] (Bertrand and Pittenger, 1968). There were some difficulties encountered with [abn-1] and [abn-2]: the growth rate of the heterokaryons varied and many cultures did not survive serial transfer; however, a few cultures were isolated which possessed nuclear markers of the wild type strain and an abnormal growth phenotype (Garn-
Figure 3. Heterokaryon Formation.

A represents a slow growth mutant with abnormal cytoplasmic factors (▲) and unmarked nuclei (○). B represents a strain with a normal growth phenotype. It has normal cytoplasmic factors (Δ) and marked nuclei (●). C is the heterokaryon with developing conidia. D is part of the mycelium resulting from one of the conidia. It contains the abnormal cytoplasmic factors with marked nuclei. Adapted from Suzuki and Griffiths, 1976.
Some of these cytoplasmic growth mutants of *N. crassa* are female sterile ([abn-1] and [abn-2]). They are thought to be cytoplasmic mutants because, when they are used as the male parent their phenotype is not passed on as would be expected of a nuclear mutation. Also, in these cases heterokaryotic transfer is often used as an alternative indication of an extranuclear cause of erratic growth.

When heterokaryons were obtained involving cytoplasmic growth mutants of *N. crassa*, the strains used were known to be compatible with respect to vegetative heterokaryosis. In the case of *N. intermedia*, however, the heterokaryon incompatibility system has not yet been researched. Consequently, strains have not been identified as possessing certain incompatibility genes (if they do exist).

Once extranuclear inheritance is determined, there are several possible causes. First, the trait could be carried in the genetic information of an organelle such as the mitochondrion or, in the case of green plants, the chloroplast. This possibility is discussed in Section II. Second, the unusual phenotype could be the result of a viral infection.

A disease (known as "die-back") of the common cultivated mushroom and characterized by slow, abnormal growth, is caused by a virus (Hollings *et al.*, 1963). More significantly, virus-like particles (VLP) have been found associated with three slow growing strains of *N. crassa*, one of which was a natural isolate (Tuveson and Peterson, 1972). These VLP are polyhedral particles with a diameter of 170 nm. Tuveson and Peterson examined [poky], [abn-1], and another variant strain designated P147, as well as a wild type strain. D.D. Perkins
had collected strain P147 in Indonesia and noted that it grew slowly even upon the initial isolation. All three slow growing strains had VLP associated with them, the wild type* did not. The researchers did not claim that these VLP necessarily caused the abnormal growth phenotype, since they did not show infectivity of the particles. Such particles would, however, be expected to show cytoplasmic inheritance, just as the unusual growth habit does.

To explore the inheritance and cause of the erratic growth of the *N. intermedia* isolates, they were: 1) put through reciprocal crosses with normal isolates, 2) tested for heterokaryotic transfer, 3) examined for the presence of virus-like particles, and 4) characterized with respect to several biochemical traits (Section II).

* Strain P147 can be considered a wild type strain since it is a natural isolate. However, any such strain (ie. a natural isolate which shows abnormal growth behavior) will be referred to as aberrant or variant. Wild type will only be used to describe strains which do not show any unusual growth characteristics.
MATERIALS AND METHODS

Strains

*Neurospora intermedia* strains 2360, 2361, 2363, and 2366 are natural isolates originally collected from Kauai, Hawaii, by D.D. Perkins. They were obtained from the Fungal Genetics Stock Center (FGSC), Arcata, California, and are listed in Table I along with other *N. intermedia* strains tested. An additional 83 isolates also from Kauai, were donated by Perkins and are listed in Table II. *Neurospora intermedia* FGSC auxotrophic strains 3386 and 3401 were used for some crosses. A histidine requiring mutation was induced into strain 2360 by D.L. Robbins using ultraviolet light. This strain is abbreviated 2360his. Other strains used for the heterokaryon tests included *N. crassa* and *N. intermedia* auxotrophs obtained from the FGSC and listed in Table III.

The only strain examined for virus-like particles was FGSC strain 2360.

Media and Growth Conditions

Minimal Vogel's, Nutritional Testing, Plating, and Crossing media are all described by Davis and deSerres (1970). Complete medium (containing 2% Vogel's solution, 0.005% tryptophane, 0.5% casein hydrolysate, 0.5% yeast extract, 1% dextrose, 1% vitamin solution, and 2% agar) was used for some tests. Supplementation consisted of 0.25 mg/ml for histidine, methionine, leucine, and cysteine; and 0.05 mg/ml for pantothenic acid, inositol, and riboflavin. Water and acetone washed agar was used for heterokaryon tests to assure the absence of extraneous nutrients.
Table I. *Neurospora intermedia* isolates obtained from the FGSC.

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Pen. = Peninsula
Table II. *Neurospora intermedia* isolates obtained from D.D. Perkins.
(all from Kauai, Hawaii).

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<td>P 614</td>
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</tr>
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<td>A</td>
<td>P 617</td>
<td>A</td>
<td>P 794</td>
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</tr>
<tr>
<td>P 568</td>
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<td>P 618</td>
<td>a</td>
<td>P 795</td>
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<td>P 619</td>
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<td>P 796</td>
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<td>P 622</td>
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<td>P 799</td>
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</tr>
<tr>
<td>P 576</td>
<td>A</td>
<td>P 623</td>
<td>A</td>
<td>P :800</td>
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</tr>
<tr>
<td>P 577</td>
<td>A</td>
<td>P 624</td>
<td>A</td>
<td>P 801</td>
<td>a</td>
</tr>
<tr>
<td>P 587</td>
<td>A</td>
<td>P 625</td>
<td>A</td>
<td>P 802</td>
<td>a</td>
</tr>
<tr>
<td>P 591</td>
<td>A</td>
<td>P 626</td>
<td>A and a</td>
<td>P 803</td>
<td>A</td>
</tr>
<tr>
<td>P 592</td>
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<td>P 628</td>
<td>a</td>
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<td>P 630</td>
<td>a</td>
<td>P 805</td>
<td>A</td>
</tr>
<tr>
<td>P 594</td>
<td>A</td>
<td>P 631</td>
<td>A and a</td>
<td>P 806</td>
<td>A</td>
</tr>
<tr>
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<td>P 632</td>
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<td>P 634</td>
<td>a</td>
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</tr>
<tr>
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<td>A</td>
<td>P 635</td>
<td>a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P 599</td>
<td>a</td>
<td>P 636</td>
<td>A and a</td>
<td></td>
<td></td>
</tr>
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<td>P 602</td>
<td>a</td>
<td>P 638</td>
<td>a</td>
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<td></td>
</tr>
<tr>
<td>P 603</td>
<td>a</td>
<td>P 639</td>
<td>a</td>
<td></td>
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</tr>
<tr>
<td>P 604</td>
<td>A</td>
<td>P 640</td>
<td>A and a</td>
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<td></td>
</tr>
<tr>
<td>P 605</td>
<td>A</td>
<td>P 641</td>
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</tr>
<tr>
<td>P 606</td>
<td>A</td>
<td>P 643</td>
<td>A and a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P 607</td>
<td>A and a</td>
<td>P 644</td>
<td>A and a</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Note that none were of interest to this study.*
Table III. *N. crassa* and *N. intermedia* auxotrophic strains used for Heterokaryon Testing.

<table>
<thead>
<tr>
<th>FGSC number</th>
<th>het genes</th>
<th>auxotrophic marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>1423</td>
<td>CDE</td>
<td>pan</td>
</tr>
<tr>
<td>1424</td>
<td>CdE</td>
<td>pan</td>
</tr>
<tr>
<td>1425</td>
<td>cDE</td>
<td>pan</td>
</tr>
<tr>
<td>1426</td>
<td>cdE</td>
<td>pan</td>
</tr>
<tr>
<td>1454</td>
<td>CDe</td>
<td>inos</td>
</tr>
<tr>
<td>1453</td>
<td>Cde</td>
<td>inos</td>
</tr>
<tr>
<td>1455</td>
<td>cDe</td>
<td>inos</td>
</tr>
<tr>
<td>1422</td>
<td>cde</td>
<td>inos</td>
</tr>
<tr>
<td>478</td>
<td>CDE</td>
<td>rib-2</td>
</tr>
<tr>
<td>476</td>
<td>cDE</td>
<td>inos</td>
</tr>
<tr>
<td>538</td>
<td>CdE</td>
<td>inos</td>
</tr>
<tr>
<td>474</td>
<td>cdE</td>
<td>inos</td>
</tr>
</tbody>
</table>

* all strains are mating A

<table>
<thead>
<tr>
<th>FGSC number</th>
<th>auxotrophic marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>3370</td>
<td>arg</td>
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<tr>
<td>3378</td>
<td>arg</td>
</tr>
<tr>
<td>3389</td>
<td>cys</td>
</tr>
<tr>
<td>3395</td>
<td>his(^\Delta)</td>
</tr>
<tr>
<td>3397</td>
<td>his(^\Delta)</td>
</tr>
<tr>
<td>3399</td>
<td>his(^\Delta)</td>
</tr>
<tr>
<td>3393</td>
<td>asn</td>
</tr>
<tr>
<td>3391</td>
<td>his(^\Delta)</td>
</tr>
</tbody>
</table>

\(^\Delta\)This his locus is a different one from the 2360his locus (ie. at least one of these strains would possess a different his locus from that of 2360his).

Auxotrophic requirement abbreviations:
- pan-pantothenic acid
- inos-inositol
- rib-riboflavin
- arg-arginine
- cys-cysteine
- his-histidine
Strain 2360 was grown in liquid media consisting of minimal Vogel's medium without agar, in preparation for electron microscopy. The sucrose buffer used for harvesting the mycelia was 0.44M sucrose, 10mM Tris-HCl pH 7.2, and 5mM EDTA (ethylenediamine-tetraacetic acid disodium salt).

Growth rate tests were carried out in 50 cm. growth tubes containing 30 mls. of Vogel's medium, at room temperature. All other culture and crosses were grown at 25°C. Any strain that showed a stop-start growth pattern was also grown in the 25°C incubator to confirm its erratic pattern.

Methods
A. Growth Rate Tests

Growth rate measurements involved inoculating a culture at one end of a growth tube, and marking its daily progress until it reached the opposite tube end. Variant strains that never attained this goal were allowed a maximum of 39 days to ensure, within reason, that they would not resume growth. Once strains possessing an abnormal growth phenotype had been identified, conidia suspended in 0.1% agar were plated on petri dishes using an overlayer technique (Newmeyer, 1954). For each strain a minimum of thirty-five colonies, presumably arising from a single conidium, were isolated in an attempt to obtain more homogeneous cultures. These were put in growth tubes, and one isolate showing an erratic pattern was selected for each strain, to be used for genetic analysis and biochemical studies (Section II). All the anomalous strains were also tested on complete media in growth tubes.

The abnormal strains were put through reciprocal crosses to either normal strain 2361 (mating type a) or 2366 (mating type A).
These two strains are also from Kauai. Each cross was simultaneously replicated 20 times. Reciprocal crosses are unusually straightforward in *Neurospora*, since either mating type (A or a) can be used as the maternal or paternal parent. The maternal parent is that which is initially inoculated onto crossing medium. These are fertilized 7-10 days later by a conidial solution of the strain acting as the paternal parent. After 21 days either random spores or tetrads are isolated, heat shocked for 30 minutes at 60°C to initiate germination, and grown at 25°C.

B. Heterokaryon Tests

The initial step in forcing heterokaryons is to grow the strains on appropriately supplemented vegetative media. After 4-5 days growth, a conidial suspension is prepared containing approximately $10^4$ conidia per ml. of distilled water. A single drop of the suspension is placed into test tubes of minimal media. Added to this is a drop of the suspension from another strain with a different auxotrophic marker. Each strain is also inoculated singly to test for "leakiness" (growth of the supposed auxotrophic strain on minimal media). Each test is repeated in five tubes. After 3, 4, and 5 days the results are recorded.

All *N. crassa* auxotrophic strains which showed a degree of leakiness, were replaced by a strain carrying the same *het* (heterokaryon incompatibility genes such as C, D, and E as previously described) genes, but a different auxotrophic mutation. Heterokaryons failed to form between 2360his and the *N. crassa* auxotrophs. At about the same time as these experiments ended, auxotrophic strains of *N. intermedia* became available through the FGSC. All these strains were tested.
When these also failed to form heterokaryons, a new avenue was explored. If strain 2360 was used as the male parent in a cross to a *N. intermedia* auxotroph, some progeny would be expected to have nuclear genes of 2360 within a normal (with respect to growth phenotype) cytoplasm. These new strains could thus possess the *het* genes of 2360, be of mating type A (as is 2360), and still show a different auxotrophic marker than strain 2360his. They would then be expected to form heterokaryons with 2360his, if the *het* genes were the same. However, this depends on whether or not the heterokaryon incompatibility system in *N. intermedia* is similar to *N. crassa*. In other words, if the incompatibility alleles must be homogenic for a heterokaryon to form. Although nothing is known about the incompatibility system of *N. intermedia* this approach was still worthy of trial. So, crosses were made using strains 2360 as the male and 3401 and 3386 as the female. 200 progeny were isolated per cross. 104 progeny grew from the cross 3401 x 2360 and 121 grew from the cross 3386 x 2360. This total of 225 isolates was tested for nutritional requirements, by spot testing on supplemented media as well as minimal media. Mating type tests were performed as described by Davis and deSerres (1970). The 43 auxotrophic mating type A isolates of the desired type were put through heterokaryon tests with 2360his.

C. Virus Search

10^6 conidia per ml. of strain 2360 was inoculated into 300 mls. of liquid media and grown at 25°C in a shaker incubator. The 24 hour old culture was harvested by suction filtration, washed with a sucrose buffer, and ground with acid washed sand in a mortar and pestle. The
preparation was centrifuged twice at 3,000 rpm to remove sand and large clumps of mycelium. The remainder of the procedure consisted of standard preparation for electron microscopy (Dawes 1971) and was carried out by Dr. Stace-Smith at Agriculture Canada, University of British Columbia.
RESULTS

Growth Characteristics of the Original Strains

Most isolates studied showed linear growth rates and attained the 50 cm. tube length in 5-8 days. Two out of 39 N. intermedia isolates obtained from the FGSC, 2360 and 2363, revealed a stop-start growth pattern. Of 83 additional strains donated by D.D. Perkins three, P804, P608, and P594, proved to be variant cultures. Thus, there was a total of five abnormal strains (plus one auxotroph, strain 2360his). The growth curves of these are shown in Figure 4. As can be seen, the cultures may stop and start growth several times, with no consistency in the duration of either the growth or stop phases. Normal growth rates are between 3.5 mm/hr and 5.3 mm/hr. The rates of abnormal strains vary, on the average, from 0 mm/hr to 5.3 mm/hr.

Colony Isolates

It was also noted that the same isolate, simultaneously put into two growth tubes, by mass transfer, will give different patterns (Figure 5). This created suspicion about the homogeneity of the culture. Therefore, the abnormal isolates were put through a purification procedure by thinly plating conidial suspensions, and picking the colonies which arise from a single conidium. Each colony was then examined with respect to its growth pattern.

All 52 isolates recovered for strain 2360, and all 37 recovered for 2363, showed erratic growth of which representative patterns are shown in Figure 6. Out of 43 colonies of P608(Figure 7), 28 came to a complete stop at least once, 10 slowed growth down to 1.6 mm/hr and
Figure 4. Growth Curves of the Five Anomalous *N. intermedius* Strains (2360, 2363, P804, P608, P594) and One Normal Strain (2361). All were grown in 50 cm. growth tubes on minimal medium at 25°C.
Figure 5. Two Growth Curves of *N. intermedia* Strain 2360. The inoculum was from the same culture tube and produces two unique growth curves when put into two growth tubes simultaneously.
Figure 6. Growth Curves of Conidial Isolates of *N. intermedia* Strains 2360 and 2363. Three representative curves are shown for each.
Figure 7. Conidial Isolates of *N. intermedia* Strain P608. Four representative growth curves of conidial isolates show the heterogeneity of this strain.
eventually resumed more normal speeds (approximately 2.3 mm/hr),
but 5 grew without slowing or stopping. However, none of the isolates
grew the tube length in less than 9 days. The colonies isolated for
strains P594 and P804 gave similar results as P608: some stopping,
some only slowing down, and others neither slowed not stopped in
the growth tube tests. Representative growth curves are given in
Figures 8 and 9. A summary of all these results is given in Table IV.
It was also noted for strain P594 that if some of the nonstopping
cultures were repeatedly subcultured they would eventually yield a
stopping strain. These stop-start strains never resumed normal growth.
They did, however, become more sickly and often died.

Reciprocal Crosses

For each strain, one of the above colonies that consistently
showed the stop-start pattern, was chosen to be analyzed genetically.
Each abnormal isolate was crossed to a normal one (see Methods) and
100 random progeny analyzed for growth rates. Two strains, 2360 and
P608, appear to be female sterile, only rarely producing perithecia
(i.e. in only 1 cross in 10). As either the male or female parent
strains P594, P804, and P608 yield approximately 1/3 to 2/3 hyalin-
colored spores, as opposed to the typical black spores. The hyalin
spores never germinated, and thus appear to be aborted.

Table V summarizes the results of all crosses. Note: 1) that
transmission of the abnormal growth behavior is very inefficient, 2)
strain 2360 as male or female yields some progeny that show "slow"
growth, that is, they never stop, but maintain a slow speed (the
highest speed is approximately 1.8 mm/hr) until reaching the full
length of the growth tube, 3) strains 2360 and P594 do show a degree
Figure 8. Conidial isolates of *N. intermedia* Strain P594. Four representative growth curves of conidial isolates show the heterogeneity of the strain.
Figure 9. Conidial Isolates of *N. intermedia* Strain P804. Four representative growth curves of conidial isolates show the heterogeneity of this strain.
Table IV. Summary of Results of Conidial Isolation for *N. intermedia*
Strains P594, P608, and P804.

<table>
<thead>
<tr>
<th>Slowest growth</th>
<th>P608</th>
<th>P594</th>
<th>P804</th>
</tr>
</thead>
<tbody>
<tr>
<td>approximately 2.3 mm/hr</td>
<td>5</td>
<td>24</td>
<td>13</td>
</tr>
<tr>
<td>Slowest growth approximately 0.8 mm/hr</td>
<td>5</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td>Slowest growth approximately 0.4 mm/hr</td>
<td>5</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Slowest growth 0.0 mm/hr</td>
<td>28</td>
<td>8</td>
<td>10</td>
</tr>
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Table V. Summary of Results of Reciprocal Crosses of the Normal and Abnormal Isolates of *N. intermedia*.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Number of progeny germinated per 100 isolated spores</th>
<th>Number of stoppers</th>
<th>Maternal Inheritance</th>
<th>Slow growers</th>
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</thead>
<tbody>
<tr>
<td>2361 x 2360</td>
<td>72</td>
<td>0</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>2360 x 2361</td>
<td>79</td>
<td>2</td>
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<td>3</td>
</tr>
<tr>
<td>2361 x 2363</td>
<td>86</td>
<td>1</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>2363 x 2361</td>
<td>53</td>
<td>2</td>
<td>*</td>
<td>0</td>
</tr>
<tr>
<td>2366 x P594</td>
<td>12^Δ</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>P594 x 2366</td>
<td>72</td>
<td>4</td>
<td>yes</td>
<td>0</td>
</tr>
<tr>
<td>2361 x P608</td>
<td>31</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>P608 x 2361</td>
<td>71</td>
<td>0</td>
<td>*</td>
<td>0</td>
</tr>
<tr>
<td>2361 x P804</td>
<td>87</td>
<td>2</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>P804 x 2361</td>
<td>58</td>
<td>2</td>
<td>*</td>
<td>0</td>
</tr>
</tbody>
</table>

^ΔOut of 200 isolated spores

*These strains do not show maternal inheritance in these crosses. However, that does not rule out cytoplasmic inheritance.

The maternal parent is written as the first strain in a cross (i.e. 2361 x 2360: 2361 is the female and 2360 is the male).

Slow growth is defined arbitrarily as not reaching the tube end in less than 12 days.
of maternal inheritance, 4) strains 2363 and P804 transmit the abnormal phenotype through either the male or female parent (but only rarely in both cases), 5) strain P608 never yields abnormal progeny, 6) out of 200 spores from the cross involving strain P594 as the male, only 12 germinated, this may be due to an incompatibility between the normal strain's cytoplasm and nuclear genes of strain P594 since this low viability was only noted when P594 was the male parent. Hence, it appears that this strain shows cytoplasmic inheritance. However, too few progeny were obtained when it is used as the male parent, to make a conclusive statement on the patterns of inheritance of this strain. This was not studied more closely.

Growth Characteristics of the Progeny

A majority of the progeny showed normal growth rates. Those progeny labelled stoppers in Table IV possess phenotypes reminiscent of their parents: they stop and start in no discernible patterns. The only progeny that do not fit into one of these two categories are the 8 offspring of strain 2360 which show slow growth as previously mentioned.

Growth on Complete Media

To rule out the possibility that the abnormal phenotype is caused by a nutritional requirement, all the original strains were grown on complete media. The growth curves are given in Figure 10. These are the same as those using minimal media.

Heterokaryon Formation

The first attempts at heterokaryon formation involving the stop-
Figure 10. Growth Curves on Complete Media. The five stop-start strains of *N. intermedia* (2360, 2363, P594, P608, P804) and one normal strain (2361) have been grown on complete media in growth tubes. Representative growth curves are given.
start strain 2360his, used *N. crassa* auxotrophic strains possessing the eight different combinations of heterokaryon incompatibility genes (ie. CDE, CDe, Cde, etc.). Every attempt at forming heterokaryons between 2360his and the *N. crassa* auxotrophs was unsuccessful. The results are listed in Table VI. The only tubes showing growth are those which contain a "leaky" strain. Each test was carried out simultaneously in 5 tubes, and the whole procedure was performed 3 times. Thus, each strain was tested with 2360his a total of 15 times.

Two compatible *N. crassa* strains (I-37-21 and 1453) were tested to assure reliability of the procedure. Five out of five tubes formed heterokaryons and neither strain revealed itself as leaky.

The *N. intermedia* auxotrophic strains tested also did not form heterokaryons with strain 2360his (Table VII). The final attempts at forcing heterokaryons with 2360his involved progeny from crosses of 2360 as the male parent and *N. intermedia* strains 3401 and 3386. Out of the 225 progeny isolated, a total of 43 were mating type A and auxotrophic mutants for either methionine or leucine. Again, all attempts at heterokaryon formation failed. The results are summarized in Table VIII.

**Virus Search**

Strain 2360 was examined for virus-like particles but no evidence of them was found. Tuveson and Peterson (1972) examined three slow growing strains of *N. crassa* and, on the first attempt, found VLP associated with each. However, they found only a few particles in [poky]. The choice of the word "few" implies a scarcity of such particles. Thus, it is possible that VLP exist in strain 2360 and are too scarce to have been
Table VI. Results of Heterokaryon Tests Between *N. intermedia* Strain 2360his and *N. orassa* Auxotrophs.

<table>
<thead>
<tr>
<th>Heterokaryon Test</th>
<th>Result</th>
<th>Control</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1423 + 2360his</td>
<td>+</td>
<td>1423</td>
<td>+</td>
</tr>
<tr>
<td>1424 + 2360his</td>
<td>+</td>
<td>1424</td>
<td>+</td>
</tr>
<tr>
<td>1425 + 2360his</td>
<td>+</td>
<td>1425</td>
<td>+</td>
</tr>
<tr>
<td>1426 + 2360his</td>
<td>+</td>
<td>1426</td>
<td>+</td>
</tr>
<tr>
<td>1454 + 2360his</td>
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<tr>
<td>1455 + 2360his</td>
<td>-</td>
<td>1455</td>
<td>-</td>
</tr>
<tr>
<td>1422 + 2360his</td>
<td>-</td>
<td>1422</td>
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<tr>
<td>1453 + 2360his</td>
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<td>1453</td>
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</tr>
<tr>
<td>478 + 2360his</td>
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<td>478</td>
<td>-</td>
</tr>
<tr>
<td>538 + 2360his</td>
<td>-</td>
<td>528</td>
<td>-</td>
</tr>
<tr>
<td>474 + 2360his</td>
<td>-</td>
<td>474</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2360his</td>
<td>-</td>
</tr>
</tbody>
</table>

+ indicates growth
- indicates absence of growth
* the *N. orassa* strains involved in these tests are leaky auxotrophs.
Table VII. Results of Heterokaryon Tests Between *N. intermedia* Strain 2360his and *N. intermedia* Auxotrophs.

<table>
<thead>
<tr>
<th>Heterokaryon Test</th>
<th>Result</th>
<th>Control</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>3370 + 2360his</td>
<td>-</td>
<td>3370</td>
<td>-</td>
</tr>
<tr>
<td>3378 + 2360his</td>
<td>-</td>
<td>3378</td>
<td>-</td>
</tr>
<tr>
<td>3389 + 2360his</td>
<td>*</td>
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<td>-</td>
</tr>
<tr>
<td>3395 + 2360his</td>
<td>-</td>
<td>3395</td>
<td>-</td>
</tr>
<tr>
<td>3397 + 2360his</td>
<td>-</td>
<td>3397</td>
<td>-</td>
</tr>
<tr>
<td>3399 + 2360his</td>
<td>-</td>
<td>3399</td>
<td>-</td>
</tr>
<tr>
<td>3393 + 2360his</td>
<td>+</td>
<td>3393</td>
<td>+</td>
</tr>
<tr>
<td>3391 + 2360his</td>
<td>-</td>
<td>3391</td>
<td>-</td>
</tr>
</tbody>
</table>

* very slight growth occurred in 3/15 tubes, but not enough for a transfer. The cultures soon died.

+ indicates growth
- indicates absence of growth
Table VIII. Summary of the Results of Heterokaryon Tests Between 2360his and Progeny of the Crosses: 3401 x 2360 and 3386 x 2360.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Number of Progeny isolated</th>
<th>Number of Progeny germinated</th>
<th>Number of Progeny of A mating type with a marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>3401 x 2360</td>
<td>200</td>
<td>104</td>
<td>19</td>
</tr>
<tr>
<td>3386 x 2360</td>
<td>200</td>
<td>121</td>
<td>24</td>
</tr>
</tbody>
</table>

NONE OF THE PROGENY OF THESE CROSSES FORMED HETEROKARYONS WITH 2360his.
found in the one attempt.
DISCUSSION

Five isolates of *N. intermedia* (strains 2360, 2363, P594, P608, P804) were found to have significantly different growth curves from those of the majority of strains. Two of these, 2360 and 2363, are homogeneous cultures as indicated by all the conidial isolates possessing the stop-start phenotype. However, the other three abnormal strains, P594, P608, and P804, did not show such homogeneity. Not only did some isolates not stop, but these same isolates when tested again could produce a different result in the growth rate test (ie. the stop-start character). This suggests both a heterogeneity of the conidia of each strain, and also a heterogeneity within a single conidium. The fact that subculturing eventually yields a homogeneous stop-start strain (noted in strain P594) may indicate some advantage of this phenotype over the normal growth behavior; or an advantage of the mutant factor over the normal factor (such as replicative advantage of the mutant DNA over normal DNA). However, it is not possible at this point to speculate what the nature of this advantage may be.

This suppressiveness of the anomalous phenotype exists when heterokaryons between *N. orassa* [abn] and normal strains are made (Garnjobst et al., 1965). Conidia arising from these heterokaryons showed various growth rates. However, even the normal cultures eventually became abnormal or died. Thus, the factor(s) responsible apparently became phenotypically suppressive. Why the abnormal phenotype becomes manifested is not known, but it appears that this is also happening with the *N. intermedia* isolates of heterogeneous origin.

It is unfortunate that two strains (P608 and 2360) tend to be
largely female sterile. The occasional successful cross is therefore rather suspect, since it takes an unusually long time to produce perithecia. This indicates that the anomalous strain allows the normal parent to contribute protoperithecia (theoretically rendering this normal strain as the actual female parent). When Mitchell and Mitchell (1952) performed crosses using [poky] as the female culture they, too, noted that protoperithecia did not form within the expected time (about five days for normal cultures). However, if they waited long enough, [poky] did produce proto perithecia. Crosses in which the [poky] strain was fertilized before protoperithecia had formed, did not pass on the [poky] character. This may explain the lack of progeny with the aberrant phenotype in crosses involving strain P608. If cytoplasmic inheritance is involved, and this strain never poses as the actual female, it would not pass on the stop-start phenotype. Strain 2360, on the other hand, does rarely (2 out of 79) pass on its phenotype when used as the female parent. The assumption then is that it can occasionally produce protoperithecia. However, it is possible that determinants from the normal male parent are being passed on to these rare abnormal progeny.

This female sterility is also seen in the N. crassa cytoplasmic mutants [abn-1] and [abn-2] (Garnjobst et al., 1965), [mi-4] (Pittenger, 1956), and [stp] (Srb, 1963). In crosses of all these female sterile strains, including the two N. intermedia isolates, the aberrant phenotype has never been transmitted when the abnormal strain poses as the male parent.

The fact that, even in the female fertile strains, the abnormal growth phenotype is rarely passed on to progeny, is in contrast to the
results found with [poky]. Mitchell and Mitchell (1952) noted that in most crosses involving [poky] as the female parent, all progeny were [poky]. The reason that the female fertile *N. intermedia* strains only rarely pass on their abnormal growth phenotype is not clear.

There are two lines of evidence that imply that a cytoplasmic factor is associated with the abnormal growth phenotype of the *N. intermedia* isolates. Firstly, as mentioned, this growth behavior is not commonly transmitted when the abnormal strain poses as the male parent. However, strains 2363 and P804 did transmit the stop-start phenotype when they were used as either the male or female parent (although in both cases very rarely). There is no explanation for this if maternal inheritance is involved since no anomalous progeny should be produced when the abnormal strain is the male parent. Secondly, a nuclear gene explanation is not suitable since none of the data show a simple Mendelian segregation ratio as expected for a single nuclear gene. Neither does a polygenic nuclear explanation appear as a likely candidate, since the progeny do not show a wide range of phenotypes. The only nonparental phenotype produced is that of 8 progeny from crosses involving strain 2360. These isolates never stop in the growth tube, but do take an unusually long time to reach the tube end (up to 13 days). Since a range of phenotypes is not observed in the progeny, a polygenic explanation would have to include a threshold effect. Mitchell and Mitchell (1952) noted that in some crosses involving [poky] mutants a number of progeny grew too slowly to be called wild type but not as slowly as [poky]. They offer no explanation for this. This result was unexpected not only because this was a nonparental phenotype, but also because in some cases [poky] had served as the
fertilizing parent, so that growth was expected to be normal.

Even with these unusual results, extranuclear inheritance is the most likely possibility, since there is no simple nuclear gene explanation. In other cases, such as [abn-1] and [abn-2] (Garnjobst et al., 1965), where the trait is not passed on at all due to female sterility (also, [mi-4] Pittenger, 1956, and [stp] Srb, 1963), cytoplasmic inheritance has been demonstrated by eliminating the possibility of nuclear inheritance. This can also be done for the *N. intermedia* strains. If the abnormality was the result of a single gene, half the progeny would be normal and half abnormal, regardless of how the cross was made. No such segregation is ever found. On the other hand, if the abnormality was the result of several nuclear genes, the progeny should show an entire range of various phenotypes. Again, this was not observed.

Further evidence of cytoplasmic inheritance for the female sterile strains [abn-1] and [abn-2], is that in heterokaryons with wild type the abnormal phenotype eventually becomes suppressive (Garnjobst et al., 1965). Also, when the cytoplasm of the [abn-2] strain was microinjected into wild type strains the resulting culture developed the abnormal character (Garnjobst et al., 1965). No microinjection studies were performed on the *N. intermedia* strains due to technical difficulty in the procedure. Therefore, to strengthen the evidence for cytoplasmic inheritance in the *N. intermedia* isolates, attempts were made at forcing heterokaryon formation with normal strains. Unfortunately, no heterokaryons were formed.

Heterokaryon incompatibility systems vary among species. In *N. sitophila* heterokaryosis depends on only 1 pair of alleles, *het* and
het$. Two $\text{het}^+$ strains will form a heterokaryon, regardless of mating type. \textit{N. tetrasperma} has absolutely no restrictions on heterokaryon formation and even depends on vegetative heterokaryons to complete its life cycle (Fincham \textit{et al.}, 1979). However, the system in \textit{N. crassa} is more stringent. There have been more than 10 incompatibility loci identified in the \textit{N. crassa} genome (Mylyk, 1975). Studies on \textit{N. crassa} populations revealed a great deal of variability in the heterokaryon genotypes of strains within a population, as well as from one population to another (Mylyk, 1976).

Consequently, it is not very surprising that heterokaryons failed to form in the \textit{N. intermedia} strains tested. They are all natural isolates, not laboratory derived strains, and thus most probably represent a vast array of genotypes all lumped under the heading of "wild type". It was, therefore, reasonable to cross the $\text{het}$ genes of the abnormal strain, 2360, into a normal cytoplasm, although the progeny of this type also failed to form heterokaryons with 2360his. Perhaps further backcrossing of these progeny to 2360 would have yielded strains with even more 2360 $\text{het}$ genes. The number of backcrosses would depend on the number of $\text{het}$ genes involved. However, the \textit{N. intermedia} incompatibility system may not depend on homogeneity of these $\text{het}$ genes and then these crosses would be futile.

It is likely that the incompatibility system is more complex than in \textit{N. sitophila} and \textit{N. tetrasperma}, since such a large number of \textit{N. intermedia} auxotrophic strains was tested. No successful \textit{N. intermedia} heterokaryons have ever been reported in the literature. It would clearly require further tests of all combinations of the \textit{N. intermedia} auxotrophs available to unravel the genetics of incompatibility in this
species. Regardless of the failure of heterokaryosis, the evidence for extranuclear inheritance was strong enough that various cytoplasmic causes were explored.

The theory that a viral infection was causing the stop-start phenotype is by no means unfounded. All five N. intermedia strains were isolated from the same Hawaiian Island, and could consequently have been exposed to the same source of viral infection. Also, as mentioned virus-like particles had previously been found associated with slow growing strains of N. orassa (Tuveson and Peterson, 1972). Since only one strain, 2360, was examined for VLP, it is possible that some or all of the other abnormal strains do possess VLP. However, the similarity of the aberrant phenotype suggests a common cause in all five strains. As mentioned, it is also possible that VLP are associated with 2360 but were not found. Other possible cytoplasmic causes are discussed in Section II.
SECTION II

Biochemical Analysis
The biochemical characterization of the *N. intermedia* isolates involved three aspects: 1) respiration and cytochrome studies, 2) an investigation of mitochondrial ribosomes, and 3) mitochondrial DNA restriction enzyme analyses. The respiratory system of wild type *N. crassa* mitochondria consists, in part, of cytochromes a, b, and c, and is cyanide, azide, and antimycin sensitive (Diacumakos et al., 1965; Haskins et al., 1954). The diagram of the electron transport chain (Figure 11) shows the different points at which it is inhibited by the various toxins (Lehninger, 1975).

In this species of *Neurospora* there are several viable respiratory-deficient mutants. The most extensively studied of these is the renowned [poky] mutation. This strain possesses an excess of cytochrome c up to 16 fold that of wild type (Haskins et al., 1954). A spectrum of the [poky] cytochromes is shown in Figure 12, along with the wild type. The cytochrome system requires the presence of cytochromes a and b. The lack of a detectable amount of these cytochromes suggests that respiration in [poky] does not totally depend on the cytochrome system. Further evidence of this was found when studies on the effects of known respiratory inhibitors showed that [poky] is mainly resistant to cyanide, azide and antimycin, while wild type is not (Tissieres et al., 1953; Lambowitz et al., 1972). For example, the addition of cyanide to wild type blocks the electron transport from cytochrome a to oxygen, yielding completely reduced cytochrome a (Lambowitz et al., 1972). [poky] is not cyanide sensitive. This points to an alternate oxidase system shunting the electrons from the
Figure 11. The Electron Transport Chain. A diagrammatic representation of the electron transport chain. The various inhibitors are shown, as well as the probable sites of ATP production. FP designates flavoproteins.

Taken from: Lehninger, 1975.
Rotenone, Amytal

\[ \text{NAD} \rightarrow \text{FP}_1 \]

\[ * \]

\[ Q \rightarrow \text{cyt b} \rightarrow \text{cyt c} \rightarrow \text{cyt aa}_3 \rightarrow O_2 \]

\[ * \]

Succinate \rightarrow \text{FP}_2

* ATP production sites.
Figure 12. Cytochrome Spectra of Two *N. crassa* Strains. A) wild type, B) poky mutation. Both are room temperature spectra taken during exponential growth. Taken from: Bertrand and Kohout, 1977.
substrate to oxygen.

Both wild type and [poky] appear to have this alternate oxidase pathway. However, in wild type it accounts for less than 10% of total respiration, unless the fungus is grown in the presence of antimycin A, cyanide, or chloramphenicol. In this case the alternate oxidase activity can be increased 20-fold (Lambowitz and Slayman, 1971). In [poky], both systems are present, the alternate oxidase being two to three times as active as the cytochrome system (Lambowitz et al., 1972).

This alternate oxidase system is blocked by salicyl hydroxamic acid (SHAM) while the cytochrome system is not (Lambowitz and Slayman, 1971). Further work with various inhibitors, and experiments in which electrons are donated directly to cytochrome c, have shown that there is no connection between the cytochromes and alternate oxidase. The evidence indicates a model of a branched electron transport system as shown in Figure 13. As can be seen the cytochromes and alternate oxidase share dehydrogenases and flavoproteins (Lambowitz et al., 1972).

Although much work has been carried out on the alternate oxidase system of many organisms, the components of this pathway are still unknown (Vanderleyden et al., 1980). However, evidence has accumulated that points to the involvement of quinones in this system (Moore and Rupp, 1978).

Cyanide-resistant respiration occurs in some higher plants (Arum James and Beevers, 1950; Symplocarpus foetidus Hackett, 1957), whose respiratory system also involves an alternate oxidase sensitive to hydroxamic acids. The model proposed by Storey and Bahr (1969) for Symplocarpus is that of a branched electron transport system similar to the one proposed for N. crassa. Several species of algae also have
Figure 13. Branched Electron Transport. The model for a branched electron transport system in mitochondria is shown. X depicts the cyanide-resistant oxidase and Y an unspecified component which can transfer electrons from the flavin step to the cyanide resistant oxidase or to the $b$ type cytochromes. Taken from: Lambowitz et al., 1972.
cyanide-insensitive respiration (Chlamydomonas reinhardtii Hammersand and Thimann, 1965; Euglena gracilis Sharples and Butow, 1970; Atasia klebsii van Dach, 1942), as well as some protozoa (Mayorella palentiae Reich, 1955; Trypanosoma vivax Ryley, 1956; Paramecium caudatum Clark, 1945), yeast (Rhodotorula glutinis Matsunaka et al., 1966) and other fungi (Myrothecium verrucarum Kidder and Goddard, 1965).

Other cytoplasmic growth mutants of N. crassa also show cytochrome spectra lacking cytochromes a and b, but with an excess of c ([exn], [stp], Bertrand and Pittenger, 1972; [abn], Diacumakos et al., 1965). The abnormal N. intermedia strains resemble these mutants in growth phenotype, as well as in showing maternal inheritance. Thus, it was of interest to study the cytochrome system of the N. intermedia isolates.

The second phase of the biochemical investigation involved the mitochondrial ribosomes. Mitochondrial ribosomes have been isolated from a variety of organisms including Neurospora, Aspergillus, yeast, rat, mouse, hamster, man, locust, Tetrahymena, Xenopus, and Euglena (Borst and Grivell, 1971). The sedimentation coefficients of these ribosomes range from 55-60S for animal cells to 70-80S for microorganisms and higher plants (Lambowitz, 1979). They are unlike cytosol ribosomes, but similar to prokaryotic ribosomes in that they are chloramphenicol sensitive and cyclohexamide resistant (Borst and Grivell, 1971; Lamb et al., 1968).

Mainly through work on the [poky] mutant, it has been found that mitochondrial ribosome assembly in N. crassa depends on nuclear as well as mitochondrial genes (LaPolla and Lambowitz, 1977). Hybridization studies show that the mitochondrial (mt) ribosomal RNA's (rRNA) are
transcribed from mt DNA while most of the mt ribosomal proteins are coded for by nuclear DNA, synthesized in the cytosol, and transported into the mitochondria (Schatz and Mason, 1974). There is, however, at least one exception. The protein designated S-5 which is associated with the mt small ribosomal subunit is synthesized within the mitochondria (Lambowitz et al., 1976).

*N. crassa*’s mt ribosomes are 73S with subunits of 50S and 37S (Kuntzel and Noll, 1967; Kuntzel, 1969). The 50S subunit is composed of 25S rRNA and various proteins. The 37S subunit consists of 19S rRNA and proteins. At one point, low molecular weight RNA (ie. 5S) was considered a universal component of ribosomes, but it has been found that there is no low molecular weight RNA (excluding tRNA) in *N. crassa* mitochondria (Lizardi and Luck, 1971). This is also known to be true for mitochondria of other fungi, as well as animal cells (Chua and Luck, 1974).

During the investigation of [poky], the mitochondrial ribosomes were found to be defective. They are deficient in small subunits (30S) when compared to wild type *N. crassa* (Rifkin and Luck, 1971). This deficiency of small subunits is accompanied by a deficiency of 19S rRNA. There is, however, no alteration of the structure of the 19S rRNA that is present (Lambowitz and Luck, 1976). LaPolla and Lambowitz (1977) proposed that the 19S rRNA is transcribed in wild type amounts and would be functional, except that it is rapidly degraded when not integrated into ribosomes. Studies involving the effect of chloramphenicol on mt ribosome assembly show that protein S-5 may be required for maturation of small subunits (LaPolla and Lambowitz, 1977). The [poky] mutant is deficient in several small subunit proteins, possibly
including S-5, and this may be the basis of the unusual phenotype since S-5 is intramitochondrially synthesized (Lambowitz et al., 1976).

The *N. intermedia* stop-start strains have been characterized with respect to growth and inheritance patterns, as well as mitochondrial respiration (see Section I and Results in Section II). However, none of these traits reveals information as to the basis of the unusual phenotype, but instead may only be manifestations. Consequently, following the history of the investigation of the [poky] mutation, research on the *N. intermedia* strains turned to analysis of mt ribosomes. Mitochondrial ribosome profiles were carried out to determine whether or not there were subunit deficiencies.

Finally, the third aspect studied was mt DNA. The first evidence for mt DNA was found by Nass and Nass (1962). The same material was also being found in other organisms (*Amoeba*, Pappas and Brandt, 1959; mouse oocyte, Parsons, 1961) and was eventually recognized as DNA. The mt DNA of animal cells is circular with a molecular weight of $10^7$ daltons, while that of higher plants is generally larger (up to about $7 \times 10^7$ daltons, Quagliarello, 1976).

Mt DNA of the yeast *Saccharomyces cerevisiae* has been shown to code for a number of mitochondrial transfer RNA's, ribosomal RNA's, and messenger RNA's. These mitochondria possess the ability to transcribe this DNA and translate the RNA into proteins (Borst, 1971). The gene products of this DNA in yeast include three of the seven subunits of cytochrome c oxidase, one of the seven subunits of the cytochrome bc$_1$ complex, and three of the ten subunits of mitochondrial ATPase (Borst and Grivell, 1978). The mitochondrial DNA of yeast has been thoroughly studied and several noteworthy discoveries have been made. This DNA
can possess deletions that remove 20-99% of its sequence. The remaining DNA of these "petite" mutants is amplified by tandem duplication so that the amount of mt DNA is the same as in the wild type (Borst and Grivell, 1978). In addition to this interesting phenomenon, the gene for large rRNA in yeast mitochondria is split by an intervening sequence (Borst et al., 1977). A map of the yeast mitochondrial genome is given in Figure 1. The same situation has recently been found in *N. crassa* (Mannella et al., 1979). mt DNA of the *N. crassa* cytoplasmic growth mutants has also been well studied (Bertrand et al., 1980).

Current mitochondrial DNA research includes mapping techniques involving the use of restriction enzymes. These enzymes cleave the mitochondrial DNA at specific sites and thus yield fragments of varying sizes which can be separated by electrophoresis. Restriction enzymes have been used to map mt DNA of *S. cerevisiae*, as well as *N. crassa* (Borst and Grivell, 1978; Terpstra et al., 1976). In *N. crassa* this technique is not only being used to map mitochondrial genes, but also to compare mutant and wild type strains. A map of the mt DNA of a wild type strain is given in Figure 14. Four stopper mutants have been shown to possess insertions or deletions in their mt DNA (Bertrand et al., 1980). Restriction enzyme analysis of the mt DNA of the [poky] mutant shows that most [poky] strains possess a wild type fragment pattern. Many, however, do show an addition (Mannella and Lambowitz, 1978) and one shows a deletion (Mannella et al., 1979). These alterations, therefore, are not the cause of the [poky] phenotype. Work is currently being carried out on all of the *N. crassa* cytoplasmic growth mutants in several laboratories, so that more information on the precise mechanism of the mutations may soon be available.
Figure 14. The Eco RI Restriction Enzyme Map of Wild Type *N. crassa* mt DNA. The positions of the eleven restriction fragments are given. A) the 25S RNA gene with an intervening sequence, B) the 19S RNA gene. Taken from: Bertrand et al., 1980.
No evidence has been found in any *N. orassa* strain that would indicate a heterogeneity of mt DNA (i.e. linear and circular etc.). The restriction maps support a circular genome (Bertrand, personal communication).

The relationship between the nuclear and mitochondrial genomes remains unclear. For example, the role of organelle genes is not necessarily obvious. In fact, mt DNA of yeast contributes only about 5% of the total mitochondrial protein (Borst and Grivell, 1978). However, a mutant completely lacking mt DNA is unable to form a functional inner mitochondrial membrane. Yet, the advantage for a cell to have two separate genetic systems is unknown. Since there are still so many unknowns regarding mt DNA, its function, its evolution, its interrelationship with nuclear DNA, work in this field is becoming more extensive.

Restriction enzymes have also been used in population analyses. Avise and coworkers (Avise et al., 1979) compared enzyme fragment patterns of mt DNA from natural populations of the mouse *Peromyscus*. They showed that the heterogeneity in mt DNA sequences can be used to estimate relatedness between individuals and populations. Clearly, this unique application of restriction enzyme analysis will be of considerable value to population biologists.

Since the abnormal *N. intermedia* strains are analogous to the stoppers of *N. orassa* in growth phenotype, it was of interest to analyze their cytochromes, mt ribosomal subunits, and mt DNA. The goal of these analyses would be to gain insight into 1) the mechanism of natural variants (i.e. how they arise and function), 2) the origin of the "slow" progeny that were found in some of the reciprocal crosses (Section I), 3) the nature of the mitochondrial genome through dissection of these new mutants, and 4) even to gain insight into the evolu-
tionary advantage of variant mt DNA. Perhaps, this could be accomplished through a thorough characterization of the factors which are known to be involved in the [poky] phenotype of *N. crassa*.
MATERIALS AND METHODS

Strains

Eight Neurospora intermedia strains were used. Three possess normal growth phenotypes: FGSC strains 2361, 2365, and 1940. The five abnormal strains were 2360, 2363, P608, and P804, and the auxotrophic strain 2360his. One conidial isolate (obtained as described in Section I), which consistently showed stop-start growth was selected for the remaining analyses of each strain. An attempt was made to use strain P594 in the remaining studies, however, this strain did not grow upon subsequent transfers, rendering it no longer usable for research. N. crassa wild type strain 74A obtained from Dr. H. Bertrand was studied for comparison of some characteristics.

Media and Growth Conditions

All cultures were grown at 25°C in either liquid or solid Vogel's medium (Davis and deSerres, 1970). Liquid cultures of $10^6$ conidia per ml. (final concentration) were grown in 800 mls. of media, in a 2 liter flask, and kept in shaker incubators at 25°C. The normal strains were harvested after 12-14 hrs. growth, while the slow growing strains were harvested after 24-48 hrs. Eco RI, Cla, and Kpn restriction enzymes were obtained from Miles Research Laboratories.

Methods

A. Cytochrome Spectra

The liquid cultures were harvested by filtration and washed with isolation buffer (0.44 M sucrose, 10 mM Tris-HCl pH 7.2, 5 mM EDTA).
The mycelia was ground with acid-washed sand, resuspended in the isolation buffer, and centrifuged at 3,000 rpm for 10 minutes in a Sorvall centrifuge at 4°C. The supernatant was centrifuged at 13,000 rpm for 30 minutes. The resulting pellet was resuspended with 2.5% deoxycholate (in 10 mM Tris-HCl pH 7.2, 5 mM EDTA, bringing it to a concentration of 1%) to clarify the solution. If the suspension was still unclear, it was sonicated with a sonic dismembrator for approximately 20 seconds. It was then centrifuged at 10,000 rpm for 10 minutes. 1 ml of the supernatant was diluted with spectra buffer (10 mM Tris-HCl pH 7.2, 5 mM EDTA), and put into each of two spectrophotometer cells. A spectrum was obtained by running oxidized vs. oxidized, then oxidized vs. reduced. A few crystals of potassium ferricyanide were used to oxidize the solutions and a few crystals of sodium dithionite were used to reduce them. Spectra were run from 650-500 nm. at the appropriate O.D. on a Beckman Acta V spectrophotometer.

B. Respiration

3 mls. of Vogel's liquid medium in which 10^6 conidia per ml. had been growing for 12-24 hrs (depending on growth phenotype) was used as respiration media. Oxygen uptake was measured with a Clark oxygen electrode at 25°C. The respiration media was saturated with air (240 μM O_2). Salicyl hydroxamic acid was dissolved in absolute ethanol, while potassium cyanide was dissolved in distilled water.

C. Mitochondrial Isolation

Mitochondria free of cytostolic ribosome contamination can be obtained by flotation gradient centrifugation, all steps being carried
out at 4°C. The suction filtered cultures were washed with cold isolation buffer (0.44 M sucrose, 10 mM Tris-HCl pH 7.6, 0.1 mM EDTA), ground with acid washed sand and resuspended with isolation buffer. The suspension was centrifuged at 4,000 rpm for 10 minutes to pellet out debris, and the supernatant was centrifuged at 13,000 rpm for 30 mins. Both spins were carried out in a Sorvall SS-34 rotor. However, if the sample was large a Sorvall GSA rotor was used and the sample was centrifuged twice at 4,500 rpm for 10 mins. and then at 13,000 rpm for 1 hr. In either case, care was taken to remove all buffer from the resulting mitochondrial pellet which was then resuspended in 60% sucrose (ultra pure sucrose in 10 mM Tris-HCl pH 7.6 and 0.1 mM EDTA was used) and brought to a total of 7 ml in an ultracentrifuge tube. This was overlayed with 2-3 ml of 55% sucrose and then 2-3 ml of 44% sucrose (both were ultrapure sucrose in 10 mM Tris-HCl pH 7.6 and 0.1 mM EDTA). These gradients were centrifuged at 39,000 rpm for 1½ hours during which time the mitochondria form a tight band between the 44% and 55% sucrose layers. Exclusion of EDTA or the addition of Mg ++ during this isolation leads to contamination by cytosolic ribosomes (Lambowitz, 1979).

D. Preparation and Analysis of Mitochondrial Ribosomes

The band of mitochondria was pipetted from the gradient to a Sorvall SS-34 centrifuge tube and diluted 1:3 with HKCTD (500 mM KCl, 50 mM CaCl₂, 25 mM Tris-HCl pH 7.6, 5 mM dithiothreitol). It has been shown that Ca ++ suppresses nuclease activity in Neurospora (Linn and Lehman, 1966), and thus it is included in the buffer at this point. The suspension was centrifuged at 12,000 rpm for 20 mins.
The pellets were resuspended with enough HKCTD to yield approximately 12 mg. of mitochondrial protein per ml. The protein estimation was carried out by precipitating a 50 μl aliquot with 500 μl of 10% TCA. If the protein concentration was correct, the solution immediately became cloudy and after 1 min. had a light precipitate. More HKCTD was added as needed.

Once the dilution was correct, puromycin (20 mM in 100 mM Tris-HCl brought to pH 7.6 with KOH and stored at -20°C, then heated for 10 mins. at 37°C before use) was added to 250 μl of mitochondria to a final concentration of 1 mM puromycin. The puromycin dissociates the ribosome monomers into subunits. This was incubated at room temperature for 10 mins. Triton-X-100 at a final concentration of 1% lyses the mitochondrial membrane. The sample was then overlayed onto sucrose gradients of ultrapure 5%-20% sucrose in HKMT (500 mM KCl, 25 mM MgCl\textsubscript{2}, 25 mM Tris-HCl pH 7.6). Mg\textsuperscript{++} is now substituted for Ca\textsuperscript{++} on the assumption that nucleases are no longer active, and since the effects Ca\textsuperscript{++} may have on the activity of puromycin is unknown. These gradients were centrifuged at 39,000 rpm for 3\frac{1}{2} hrs. in a Beckman SW 40 rotor (or its equivalent), then analyzed with a density gradient fractionator at A\textsubscript{254}.

### E. rRNA Extraction

rRNA was extracted from whole mitochondria obtained from the flotation gradient described above. The pelleted mitochondria were resuspended in 2 mls of HKCTD. In a glass homogenizer 50 mM Tris-HCl pH 7.6, 5 mM MgCl\textsubscript{2}, 1% SDS (sodium dodecyl sulfate) and 0.15 ml diethylpyrocarbonate (a nuclease inhibitor) were mixed for 2 minutes at 4°C. The mitochondria were added and homogenized for 2-3 mins. at
4°C. The homogenate was then incubated at 37°C for 5 mins. and centrifuged at 9,000 rpm for 10 mins. at 24°C. 0.5 g NaCl was added to the supernatant and the solution was kept on ice for 10 mins. It was then centrifuged at 10,000 rpm for 30 mins. at 4°C, to remove the SDS-protein precipitate. Two volumes of ice cold ethanol were added to the supernatant and it was stored overnight at -20°C to precipitate the RNA. The sample was spun for 45 mins. at approximately 1,000 rpm in a clinical centrifuge. The RNA was dissolved in a buffer of 0.04M Tris (ultapure), 0.3 mM EDTA, 22% sucrose, 0.1% SDS, 0.42% NaH₂PO₄·H₂O for electrophoresis through an agarose-acrylamide composite gel (Peacock and Dingman, 1968).

F. mt DNA Preparation and Digestion

Mitochondria were isolated by sucrose flotation gradients as described, except that all glassware was acid cleaned for this procedure. The mitochondrial band was removed from between the 44% and 55% sucrose layers, diluted 1:3 with HKCTD and spun for 30 mins. at 18,000 rpm. The supernatant was discarded, and the pellet was resuspended in 3 volumes of HKCTD with 2% (final concentration) Nonidet added to lyse the mitochondria. The suspension was centrifuged at 55,000 rpm for 16 hrs. on a 1.85 M sucrose (in HKCTD) cushion. The resulting ribonucleoprotein pellets were carefully washed with cold distilled water. At this point the samples could be frozen at -20°C until ready for use.

The pellets were resuspended in 2 mls. N-SET (100 mM NaCl, 100 mM Tris·HCl pH 8.2, 2 mM EDTA, 1% SDS). Then, 2 mls. of phenol buffer (10 volumes distilled phenol to 3.5 volumes H-NET; H-NET contains 150 mM NaCl, 100 mM Tris- HCl pH 8.2, 1 mM EDTA) were added and the samples were left at room temperature for 10 mins. They were then cen-
trifuged at 10,000 rpm for 10 mins. in a warm centrifuge. An additional 2 mls. of phenol buffer was added to the upper aqueous phase and the centrifugation was repeated. The upper phase was dialyzed overnight against 4 liters of H-NET. 50 μl RNAse solution (2 mg/ml RNAse A, 25 μl/ml RNAse T₁ in H-NET, preincubated at 80°C for 10 mins) was added to the dialysate and incubated at 37°C for 30 mins. 200 μl of protease solution (2 mg/ml Sigma protease VI in H-NET, preincubated for 1 hour at 35°C) was added and the solution was incubated for 40 mins at 37°C. At the end of all incubations 2 mls of phenol buffer were added, the samples were centrifuged, and the upper phase was dialyzed overnight against 4 liters of Final buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.1, 0.1 mM EDTA).

This dialysate was put on ice with 5 mls ice-cold ethanol and stored at -20°C for at least 24 hrs. The samples were centrifuged at 40,000 rpm for 1 hr and all the ethanol was removed. The pellets were resuspended in 50 μl L-NET (10 mM NaCl, 10 mM Tris-HCl pH 7.5, 0.1 mM EDTA) and O.D. readings were obtained to estimate 1 μgm of DNA to be loaded onto an agarose gel. To carry out digestion of the DNA a reaction mixture was used as described by the suppliers of the restriction enzyme. For Eco RI the mixture was: 2.5 μl 1 M Tris-HCl pH 7.5, 2.5 μl 0.1 M MgCl₂, 2.5 μl 0.5 M NaCl, 15.5 μl L-NET, 1.0 μl Eco RI. For Kpn the reaction mixture consisted of: 2.5 μl mercaptoethanol (0.42% in distilled water and made fresh for each use), 5μl L-NET, 8 μl Kpn restriction enzyme. The Cla digest was carried out in 2.5 μl Cla mix (100 mM Tris-HCl pH 8.0 and 100 mM MgCl₂), 16.5 μl distilled water, 4 μl Cla enzyme. The samples were digested for 4 hrs at 37°C. They were then electrophoresed on a 0.8% agarose gel at 50
volts for 15 hrs. The gel was stained with ethidium bromide (0.5 μg/ml of distilled water) for one hour.
RESULTS

Cytochrome Spectra

The *N. intermedia* strains which have a normal growth phenotype (FGSC 2361, 2365, and 1940) all possess cytochrome spectra similar to that of *N. crassa* wild types. The $\alpha$ peaks for cytochromes $b$ and $c$ are at 550 nm and 561 nm, respectively, in both species. However, the $\alpha$ peak of cytochrome $a$ is at 608 nm in *N. intermedia* rather than at 601 nm as it is in *N. crassa* (Lambowitz et al., 1972). The spectrum of strain 2361 is given in Figure 15 and is also representative of spectra for 2365 and 1940.

The spectra of strains 2360 and the related auxotrophic strain 2360his show little or no cytochrome $b$ relative to cytochrome $c$, and compared to the normally growing strains. Strains 2363 and P804 have a very small amount of cytochrome $a$ and no $b$, again relative to the amount of cytochrome $c$. The abnormal strain P608 appears to have a "wild type" spectrum. All of these spectra are shown in Figure 15. The cytochrome peaks are consistently located at the same position (if present) in all spectra (cytochrome $a$ at 608 nm, $b$ at 561 nm, and $c$ at 550 nm).

Oxygen Uptake Studies

Strains 2361 and 2360his were studied with respect to their sensitivity to respiratory inhibitors. Strain 2361 proved to be 92% cyanide sensitive, while strain 2360his was only 34% sensitive; both of these experiments were carried out only once. However, strain 2360his was 68% sensitive to SHAM (this is the average of two runs: 64% and...
Figure 15. Cytochrome Spectra of *N. intermedia* Strains. 
A) a spectrum representative of 2363 and P804, 
B) strain P608, C) a spectrum representative of 2360 and 2360his, and D) a spectrum representative of strains 2361, 2365 and 1940.
72% sensitivity). These results are shown in Figure 16.

Ribosome Profiles

All abnormal, and one normal strain, of *N. intermedia* were analyzed for mitochondrial ribosomal subunit deficiencies. Figure 17 shows the results of sucrose gradient analyses, and Table IX lists the ratios of large to small subunits. The values for ratios are means and are based on the area under the curve; units are arbitrary. The normal strain, 2361, shows both subunits and in a ratio of 2.2 : 1, large to small. This is comparable to that found in *N. crassa* wild type 74A which shows a ratio of 2.4 : 1 (Collins et al., 1979).

The stop-start strain, 2363, has a deficiency of small subunits so that the ratio of large to small is 7.3 : 1. All of the other stop-start strains show a deficiency of large subunits. The ratios vary from 1 : 2.0 to 1.1 : 1. Note that the ribosome profile for strain 2360his shows two peaks, but in different locations than the other profiles. It is probable that the gradient was inaccurate, thus changing the location of the large and small subunit peaks. Another point to note, is seen in the profile of strain 2360. This shows a small unlabelled peak which sediments more slowly than the small subunits. A similar peak found in some mutants of *N. crassa* is thought to be incomplete mt small subunits (Collins et al., 1979).

Mitochondrial Ribosomal RNA Analysis

Three strains were analyzed by gel electrophoresis to see if a deficiency of rRNA could be detected in 2360his. One strain was
Figure 16. Oxygen Uptake of Intact Cells.

The arrows indicate the time when either KCN or SHAM was added. The line labelled "a" shows normal respiration and is taken to be 100%. The line labelled "b" is inhibited respiration. The percent inhibition is calculated from the slope of the line. 1) strain 2360his, 2) strain 2360his 3) strain 2361.
Figure 17. Ribosome Profiles of *N. intermedia* Strains.  
A) strain 2361, B) 2363, C) P804, D) P608,  
E) 2360his, F) 2360. Peak 1 is the small  
subunit peak, and peak 2 is the large subunit  
peak.
Sedimentation is from left to right.
Table IX. Ratios of large to small subunits for the various *N. intermedia* strains. Values are obtained from areas under the appropriate peaks.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Ratio of Large : Small subunits</th>
</tr>
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<tbody>
<tr>
<td>2361</td>
<td>2.2 : 1 (5)</td>
</tr>
<tr>
<td>2363</td>
<td>7.3 : 1 (3)</td>
</tr>
<tr>
<td>2360</td>
<td>1 : 1.3 (3)</td>
</tr>
<tr>
<td>2360his</td>
<td>1 : 2.0 (1)</td>
</tr>
<tr>
<td>P608</td>
<td>1.1 : 1 (3)</td>
</tr>
<tr>
<td>P804</td>
<td>1 : 1.8 (2)</td>
</tr>
</tbody>
</table>

*These are average values. The number of runs for each strain is given in ( ).
N. orassa wild type 74A, which showed bands corresponding to 25S rRNA and 19S rRNA (Figure 18). N. intermedia strain 2361 also showed two clear bands corresponding to 25S and 19S rRNA. Strain 2360his, the only stop-start strain analyzed, shows almost no band in the expected location of 25S rRNA, but a heavy band for 19S rRNA. This confirms the above observation that there is a deficiency of large ribosomal subunits in this strain as mentioned above.

**DNA Restriction Enzyme Patterns**

Figure 19 shows the Eco RI restriction enzyme patterns of N. orassa wild type strain 74A and N. intermedia strain 2361 (a normal phenotype isolate). Both show eleven mt DNA fragments, seven of which comigrate. Not surprisingly, since they are unique species, some fragments are seen in different locations on the gel. The molecular weights of the fragments for all strains analyzed are given in Table X, and show that 2361 has a total molecular weight of approximately $41.5 \times 10^6$. This is comparable to the molecular weight of 74A, $40.9 \times 10^6$.

Figure 20 shows the Eco RI digest of strains 2363, P804, 2360, 2361, and 2365. The two strains with a normal phenotype (2361, 2365) show precise comigration of fragments. The only exception is that strain 2365 possesses an additional band. This band will be referred to as band E, and is not seen in 2361. Since strain 2361 shows Eco RI fragments 1-10 as does N. crassa (although not the same molecular weights), the DNA of this strain will be referred to as "standard" DNA. The additional DNA seen in the Eco RI digest of 2365, is not seen in the Kpn digest (Figure 21). There are no additional bands and all four bands present comigrate with those of 2361. This disappearance of the
Figure 18. Ribosomal RNA Analysis. A). *N. crassa* wild type 74A, B). *N. intermedia* strain 2361, C). *N. intermedia* strain 2360his, D). *N. crassa* strain 74A. All three strains show a faint band of mt DNA. Note that strain 2360his shows little 25S rRNA.
Figure 19. Eco RI Restriction Enzyme Digestion of *N. crassa* and *N. intermedia* Strains.

A). *N. crassa* wild type strain 74A,
B). *N. intermedia* strain 2360his, C).
*N. intermedia* strain 2361. The numbers 1-10 correspond to the fragments of strain 74A.
Table X. Molecular weights ($\times 10^6$) of *N. intermedia* mt DNA Eco RI fragments. The M.W. was obtained by plotting the log of the molecular weight vs. the distance from the well for strain 74A (molecular weights obtained from Terpstra et al., 1976) and then extrapolating the weights for the *N. intermedia* strains.

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<td>43.62</td>
<td>46.92</td>
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* The values for fragments 7a and 7b are approximate since they migrate so close together.
extra DNA may indicate that the DNA of band E comigrates with another Kpn band. It is also possible that this DNA is shredded by Kpn, and hence is not visible.

The Eco RI patterns of stop-start strain 2363 show "standard" DNA, as well as two additional bands. These bands are labelled B and D. There is no band E present. The Kpn digest shows that this strain may have an extra Kpn restriction site in band 2. One of the resulting fragments would be the second band that appears in this digest, and the other fragment would be too small to be seen. The Kpn digest shows another band not seen in strain 2361. This may correspond to the extra DNA seen in the Eco RI digest. The same situation is seen in the Cla digest of this strain (Figure 22). The fragment pattern shows additional DNA with respect to the "standard" DNA.

The Eco RI digest pattern of strain P804 shows several obvious variations from the pattern of standard DNA. Band D is present in this strain, as well as a band unique to this strain, band C. The Kpn digest also shows a very faint band which comigrates with one of the extra Kpn bands of strain 2363. Eco RI band 3 is not seen in strain P804 and may represent a deletion or may be migrating close to Eco RI band 2 (there is a faint band between Eco RI-1 and-2). Also, a band between Eco RI fragments 4 and 5 (band C) is highly amplified in this strain with respect to its other bands and may represent a plasmid (see Discussion). The DNA of this band is also probably shredded in the Kpn digest, since it is not seen. However, it may be showing up in the Cla digest as band 6 which appears somewhat amplified.
Figure 22. Cla Restriction Enzyme Digest of *N. intermedia* Strains. A). P804, B). 2361, C). 2363. The numbers 1-7 correspond to the fragments of strain 2361.
Abnormal strain 2360 has "standard" DNA, as well as the additional bands A, D, and E. This extra DNA is seen in the Kpn digest as well. Band E is the same band seen in strain 2365.

The uncut DNA shows only one band (with some smearing) in each strain (Figure 23). Thus, all three abnormal strains (2363, P804, and 2360) show additional DNA with respect to the "standard". Of this additional DNA, the abnormal strains all possess a common fragment, band D of M.W. $2.4 \times 10^6$, in their Eco RI digestion patterns that is not seen in either of the normal strains tested.
Figure 23. Uncut DNA of the *N. intermedia* Strains.
The uncut DNA of each strain (2361, 2363, P804, 2365, 2360) is shown in slots between the Eco RI digested DNA. There are no differences seen in the migration of uncut DNA and only one band is seen for each strain.
DISCUSSION

As would be expected if there is a common cause of unusual growth phenotypes for both species, most of the abnormal \textit{N. intermedia} strains (2363, 2360, 2360his, and P804) possess cytochrome spectra reminiscent of the cytoplasmic mutants of \textit{N. crassa} ([poky], [exn], [stp], Bertrand and Pittenger, 1972; [abn], Diacumakos \textit{et al.}, 1965). There is little or no cytochrome \textit{a} or \textit{b} present, and an excess of cytochrome \textit{c} relative to the amounts of \textit{a} and \textit{b}. However, the cytochrome spectrum of strain P608 showed all cytochromes present and in proportions similar to the normally growing strains 2361, 2365, and 1940.

In Section I it was noted that P608 never passes its abnormal growth phenotype on to its progeny. This was actually expected, though, if cytoplasmic inheritance is involved since the strain is female sterile. The surprising result was that it shows a normal cytochrome spectrum. This indicates a difference between P608 and the other abnormal strains which do show a lack of cytochromes \textit{a} and \textit{b}. However, P608 does show a deficiency of large mt ribosome subunits as do most of the other stop-start strains.

Abnormal cytochrome spectra may strengthen the evidence for cytoplasmic inheritance since the mitochondrion is known to code for sub-units of cytochrome oxidase and may code for other proteins involved in the cytochrome system. However, a cytochrome spectrum cannot prove or disprove maternal inheritance. There are nuclear mutants of \textit{N. crassa} which show a lack of certain cytochromes (strains C-115 and C-117, Mitchell \textit{et al.}, 1953). These two strains also show a growth phenotype
similar to [poky]. The same is true in yeast; there are both nu-
clear and cytoplasmic cytochrome-deficient mutants (Ephrussi, 1953).

Thus, the cytochrome spectra of the abnormal \textit{N. intermedia}
strains demonstrates two points: 1) that most of the erratic growth
habit strains (2363, 2360, 2360his and P804) resemble the [poky]
mutant of \textit{N. crassa} in one more characteristic, and 2) that the abnor-
mal \textit{N. intermedia} strains are not all identical.

Respiration studies also show a striking similarity between
strain 2360his and [poky]. Both strains respire, at least in part, by
a cyanide resistant pathway. This insensitivity to cyanide, and the
lack of cytochromes \textit{a} and \textit{b}, point to the involvement of an alternate
oxidase in the respiratory chain of strain 2360his. While further
work is needed to elucidate the nature of the alternate oxidase, if
it does exist, it is probable that it will resemble that which is
found in [poky]. The original work with [poky] supported the theory
that flavoproteins act as alternate oxidases. There is twice as
much flavin in [poky] as in wild type and nearly all is found as
FAD (Tissieres \textit{et al.}, 1953). However, further work then indicated
an iron-sulphur species as the alternate oxidase (Bendall and Bonner,
1971), and finally the current hypothesis is that a quinone acts as
the alternate oxidase (Moore and Rupp, 1978). While the work on the
\textit{N. intermedia} slow growing strains does not add information on the
properties of the alternate oxidase, it does increase the number of
known respiratory variants. It also shows that they can exist in n
nature, at least in \textit{Neurospora}.

The results indicate that the aberrant growth phenotype of strains
2360, 2360his, 2363, P804 and P608 is related to a deficiency of one
of the mitochondrial ribosomal subunits. The large subunit deficient strains show that a large subunit defect can have a normal cytochrome spectrum (as in strain P608) or an abnormal spectrum (as seen in strains 2360his, 2360 and P804). While the nature of the particular defect of the mt ribosomes remains unclear, it is expected to show maternal inheritance if it is coded for by mitochondrial genes. So far two defects of the stop-start strains have been associated with mitochondria: the abnormal cytochrome system and the deficiencies of mt ribosomal subunits. If the defect were within the genetic material of the mitochondria, it would show cytoplasmic inheritance. Since it is known that in N. crassa genes for mt rRNA are located within the mitochondrial genome, these are likely suspects for the defect. An abnormality in the rRNA would probably give rise to a defect in the ribosomal subunits. However, electrophoretic studies on [poky] showed that, although there was a deficiency of 19S rRNA (corresponding to the 37S subunit deficiency), there was no difference in the stoichiometry of 19S rRNA of wild type and [poky] (Lambowitz and Luck, 1976). Thus, a mitochondrially coded protein, such as S-5, is more likely to be the basis of subunit deficiency, and therefore of the unusual phenotype in the N. crassa cytoplasmic mutants.

Only one N. intermedia strain, 2363, shows a deficiency of small subunits. In this respect it is similar to [poky]. Several other cytoplasmic mutants of N. crassa have also been analyzed and all show poky-like characters: a small subunit deficiency with 19S rRNA being rapidly degraded (Collins et al., 1979). Further work on strain 2363 is necessary to see if it, too, shows 19S rRNA degradation.
The remaining stop-start strains all show a deficiency of large subunits, although to varying degrees (see Results). No other maternally inherited mutations in *Neurospora* have been shown to possess large subunit deficiencies. However, three nuclear mutants of *N. crassa* which possess abnormal cytochrome spectra are large subunit deficient. They also have a decreased ratio of 25S to 19S RNA when compared to wild type (Collins *et al.*, 1979).

The rRNA analysis of *N. intermedia* large subunit deficient strain 2360his shows very little 25S rRNA. It is probable that this situation is analogous to [poky], in that the 25S rRNA is rapidly degraded (as is the 19S rRNA in [poky]) when mature large subunits are not formed. If this is the case, it would suggest another mitochondrial ribosomal protein is coded for by the mitochondrial DNA. However, other possibilities still exist, such as a defect in the 25S rRNA itself, that cannot be ruled out. Clearly, all of these large subunit deficient strains are worthy of continued research.

The results of these ribosome analyses have shown strain P608 to be similar to the majority of stop-start strains (2360, 2360his, and P804). The reason P608 shows a similarity to these other abnormal strains in its ribosome profile and not in its cytochrome spectrum is unclear. It is also interesting that strain 2363 shows the typical cytochrome spectrum for these strains but shows a different ribosome profile. Thus, the abnormal strains, at this point can be put into three groups with respect to cytochrome spectra and ribosome profiles. Group I includes strain 2363 which lacks cytochromes a and b, and has a deficiency of small mt ribosome subunits. Group II includes P608 which shows a normal cytochrome spectrum but a deficiency of large mt
ribosome subunits. Group III consists of the remaining abnormal strains: 2360, 2360his and P804. These lack cytochromes a and b and large mt ribosome subunits. Only Group I (2363) is truly similar to [poky].

The analysis of mitochondrial ribosomes reinforced the evidence for cytoplasmic inheritance in the stop-start strains of *N. intermedia*. It also added to the list of similarities between these strains and [poky] (i.e. they all have a ribosomal subunit deficiency). However, it also revealed a difference not only between some of the strains and [poky], but also between the strains themselves. Thus, two characteristics show differences among the abnormal strains (i.e. cytochrome spectra and ribosome profiles). All of these *N. intermedia* strains were collected from one Hawaiian Island, and all possess a similar growth phenotype. Yet, the genetic cause of their phenotype may not be identical. Therefore, the remainder of this work was devoted to investigating the mitochondrial DNA. Any major alterations of the DNA of the anomalous strains should be identifiable by restriction enzyme analysis. Also, studies of this type have been carried out on the *N. orassa* growth mutants so that they were available for comparison.

The present theory regarding the growth phenotype of the *N. orassa* stopper mutants requires the existence of two species of mt DNA (Bertrand *et al.*, 1980). One species would be mutant DNA which, when accumulated in the hyphal tip, would cause cessation of growth. The other nonmutant DNA species would eventually take over and growth would resume. This theory calls for some form of replicative advantage for the mutant DNA, but the details of this are speculative. Re-
striction enzyme analysis added credit to this theory by showing that two types of mt DNA may exist in these mutants. Certain wild type bands are amplified with respect to the other bands on the gels. This indicates that these fragments are present in both the mutant DNA and the more intact DNA. In other words, two types of mt DNA are present in a cell. The mutant type contains only certain wild type bands (the amplified ones, amplified because they are present in both types of DNA) and is missing others (the nonamplified ones). The other type of mt DNA contains all (or most) of the wild type bands. Thus, when the mutant DNA accumulates the hyphae stops growing. When the intact DNA takes over growth would resume.

The phenotype of the *N. intermedia* isolates so closely resembles that of the *N. orassa* [stp] mutants, that it seemed probable they would have an analogous cause. Thus, major additions to, and deletions of, the mt DNA were looked for in the stop-start strains. In a restriction enzyme pattern a deletion of mt DNA is expected to shift a band to a new position further down the gel. An addition, on the other hand, would increase the molecular weight of the fragment and thus keep the band from migrating as far. It is not expected that an entire band will either be lost or added without affecting some other band. For this to happen the deletion would have to be the precise size of the fragment. Also, an addition would have to occur exactly between two other fragments without disturbing the restriction enzyme recognition sites. These types of deletions and additions are highly unlikely.

Two of the *N. intermedia* abnormal isolates (2360 and 2363) appear not to have any mt DNA deletions. The "standard" DNA of strain 2361
is present in both strains as seen in the Eco RI digest patterns. The only strain which does have a band missing is P804 (ie. Eco RI-3). However, there are no new bands seen that add up to the molecular weight of the missing fragment. Also, there is no evidence for a deletion in P804 in the Kpn digest. Therefore this Eco RI-3 fragment may have a slight addition causing it to migrate between Eco RI -1 and -2.

There is also no evidence of additions to the mt DNA of the abnormal strains (except as mentioned in strain P804). While there is definitely more DNA in these strains, it does not appear to be inserted into the mt DNA genome since none of the standard bands have been altered. Therefore, it is more probably "extrachromosomal" (ie. plasmid-like DNA). Plasmid-like DNA exists in mitochondria of cytoplasmic male-sterile maize (Kim et al., 1979), as well as in wild type strains of N. crassa (Collins et al., in press).

Strain P804 shows a very amplified Eco RI band which is not present in "standard" DNA. This DNA most likely exists as several tandem repeats giving the band its intensity. It is not clear why this extra DNA of P804 and the extra fragments of strains 2360, 2363 and P804 do not show up as separate bands in the undigested DNA. It is assumed that they are too large to be separated from the rest of the mt DNA since this is the case in N. crassa: plasmids occur as large pieces with tandem repeats and are not separated from the rest of the mt DNA when the undigested DNA is electrophoresed (Bertrand, personal communication).

Band E cannot be associated with the abnormal growth habit of strain 2360 since it is also found in the normal strain, 2365. How-
ever, band D is found in all of the stop-start strains, and not in either of the normal strains. It is possible that this DNA is related to the abnormal phenotype.

The stopper hypothesis for *N. crassa* requires the existence of two species of mt DNA. The *N. intermedia* isolates did not show additions or deletions (with the exception, possibly, of strain P804) within their mt DNA (although they do possess extra DNA intra-mitochondrially). However, this is not necessarily inconsistent with the *N. crassa* hypothesis. It is possible for even a point mutation to cause stopping. This would not be detectable through restriction enzyme analysis. Thus, it cannot be concluded that the extra DNA causes the erratic growth patterns of the *N. intermedia* strains. Yet, it is interesting that all the stop-start strains possess band D in their Eco RI digests. Further work on the genetic function of this DNA is sure to be profitable.

Another area which would be interesting for further studies is the difference between two normal strains. Strain 2365 contains an additional Eco RI band which is obviously nonessential for normal growth since 2361 does not possess it. Strains 2361 and 2365 are not true controls for the restriction enzyme patterns of the anomalous strains. Since all the *N. intermedia* strains were obtained from nature there are no true controls available. This would require normal strains from which the anomalous isolates were derived. Strain 2361, does however, give an indication of what DNA is required for normal growth.

The restriction enzyme patterns suggest the possibility of plasmid-like DNA within the mitochondria of the stop-start strains. This leaves open to investigation the genetic function of this DNA.
These strains of *N. intermedia* are fascinating tools of research. They show many characteristics similar to the laboratory derived mutations in the closely related species *N. crassa*. They possess an abnormal growth phenotype which appears to be cytoplasmically inherited. They show an excess of cytochrome c and lack of cytochromes a and/or b. At least one is cyanide resistant. They show defects in their mt ribosomal subunits (in one the small subunit, in the others the large subunit). They also have additional DNA independent of "standard" mt DNA. A summary of the characters studied and the results obtained is given in Table XI. This characterization has revealed much information about these strains. Yet, there is considerable room for more work. The two areas which would be most interesting and probably the most profitable for further studies are the mt ribosomes and mt DNA. Perhaps a protein analogous to S-5, which is associated with small mt ribosomal subunits in *N. crassa* and is intramitochondrially synthesized, could be found for the large subunits.

Studies on the mt DNA will indeed be informative. There is much to be learned about the mechanisms behind this unusual growth phenotype. These strains are unique in that they are natural isolates. It will be interesting to be able to hypothesize how these isolates arose and have survived while in competition with normally growing isolates.
Table XI. A Summary of the Results of the Characterization of the \textit{N. intermedia} isolates.

Transmission of growth phenotype to progeny

<table>
<thead>
<tr>
<th>Strain</th>
<th>when male parent</th>
<th>when female parent</th>
<th>produces variable conidia</th>
<th>cytochromes present</th>
</tr>
</thead>
<tbody>
<tr>
<td>2360</td>
<td>no</td>
<td>yes*</td>
<td>no</td>
<td>c</td>
</tr>
<tr>
<td>2363</td>
<td>yes*</td>
<td>yes*</td>
<td>no</td>
<td>c</td>
</tr>
<tr>
<td>P594</td>
<td>no</td>
<td>yes*</td>
<td>yes</td>
<td>----</td>
</tr>
<tr>
<td>P608</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>a,b,c</td>
</tr>
<tr>
<td>P804</td>
<td>yes*</td>
<td>yes*</td>
<td>yes</td>
<td>c</td>
</tr>
</tbody>
</table>

*in all these cases, only a few abnormal progeny were produced.

mt ribosomal subunit deficiency

<table>
<thead>
<tr>
<th>Strain</th>
<th>large subunit</th>
<th>small subunit</th>
<th>rRNA analysis</th>
<th>mt DNA analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>2360</td>
<td>yes</td>
<td>no</td>
<td>25S deficient</td>
<td>band D present</td>
</tr>
<tr>
<td>2363</td>
<td>no</td>
<td>yes</td>
<td>---</td>
<td>band D present</td>
</tr>
<tr>
<td>P594</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>P608</td>
<td>yes</td>
<td>no</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>P804</td>
<td>yes</td>
<td>no</td>
<td>---</td>
<td>band D present</td>
</tr>
</tbody>
</table>

Remarks

2360 possesses extra mt DNA which may be in the form of a plasmid.

2363 possesses extra mt DNA which may be in the form of a plasmid.

P594 does show maternal inheritance of growth phenotype.

P608 has normal spectrum, yet shows abnormal growth and a deficiency of large mt ribosomal subunits.

P804 possesses extra mt DNA which may be in the form of a plasmid with a tandem repeat.
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