FARRIOR, JULIAN WADE. Cytochrome Analysis of a Cytoplasmic Mutant of Neurospora crassa. (1971) Directed by: Dr. William K. Bates and Dr. James F. Wilson. Pp. 80.

It has been shown that mitochondria extracted from a cytoplasmic mutant, [mi-1], of Neurospora crassa produce a phenotypic change when micro-injected into a wild type strain, even if the mitochondrial donor and recipient differ in heterocaryon genotype. The change manifests itself as a conversion of the cytochrome pattern from wild type to [mi-1], and a reduction in growth rate. The change is not immediate but appears after several serial transfers of the injected strain.

Experimental results show that this change is not a gradual one but happens with an abrupt disappearance of cytochrome a-a_3 and b, accompanied by an increase in cytochrome c. The transition stage has an amount of cytochrome c intermediate between that of the wild type cell and that of the [mi-1] cell.

The wild type strain, the control strain, and the micro-injected strain have, initially, the same amount of cytochrome c per unit weight protein. The micro-injected strain shows a wild type cytochrome pattern and rate of growth through successive transfers until an intermediate stage of development is reached. At this stage, it shows an [mi-1] growth rate, but has less cytochrome c than the final stage. The final change, which comes on rapidly, produces a strain with [mi-1] cytochrome pattern, slow rate growth, and approximately twice the concentration of cytochrome c of the wild type strain. The final concentration of cytochrome c in the micro-injected

strain does not ever reach the amount present in the [mi-1] strain from which the mitochondria were originally obtained. Heterocaryon incompatibility may be a factor in this failure to reach [mi-1] cytochrome c concentration.

CYTOCHROME ANALYSIS OF A CYTOPLASMIC MUTANT OF *NEUROSPORA CRASSA*

by

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CHAPTER I

INTRODUCTION

Much insight into the structure and function of genes has been obtained through the comparison of the wild type gene with its mutant allele. The only mutations that could be studied were those that resulted in identifiable phenotypic changes (markers). Nuclear chromosomes have been well characterized through those genetic markers which occurred as either the primary or secondary effects of mutation.

Cytoplasmic inheritance has not been characterized as clearly as nuclear inheritance due to a shortage of available mutants and a lack of suitable genetic markers. Various cytoplasmic mutants of the filamentous fungus Neurospora crassa have provided some insight into cytoplasmic inheritance. The phenotypic expression of these mutations was typically a changed pattern of growth and a change in cytochrome concentration and distribution due to alterations in the mitochondria. By the use of these characteristic markers, successive stages may be observed in the phenotypic change produced in wild type cells by the micro-injection of [mi-1] mitochondria.

This present work can be placed in better perspective by a survey of the earlier work with cytochromes and cytochrome-deficient strains of fungi.

Discovery and Characterization of the Cytochrome System

Cytochromes were first observed with a spectroscope by MacMunn (1886). In tissues ranging from human to those of sponges, he found a characteristic absorption spectrum belonging to a pigment which he described under the names of myohaematin (pigment found in muscles) and histohaematin (identical pigment found in other tissues). On the properties of histohaematin, MacMunn stated that absorption bands were observed when the pigment was in a reduced state and not observed in the oxidized state. This pigment in the reduced state showed four absorption bands.

Keilin (1925) found MacMunn's names myochaematin and histohaematin misleading when applied to the respiratory pigment. He proposed the term cytochrome for the pigment signifying cellular pigment. Keilin found that cytochrome was not a single substance, as believed by MacMunn, but a system composed of three haemochromogen compounds: cytochrome a, cytochrome b, and cytochrome c, which were very different in their structures and properties.

Through his work with cytochromes in yeast and bacteria, Keilin: (a) established their intracellular localization; (b) observed their oxidation and reduction within living, intact cells, thus marshalling evidence to prove without a doubt the respiratory function of cytochromes; (c) determined the effect of different factors such as cyanide and narcotics on their biological activity; (d) determined the properties and respiratory activities of cytochromes a, b, and c with

a absorbing at the longest wave length and c at the shortest.

Studying the wing muscles of bees with the Hartridge reversion microspectroscope, Keilin found the positions of maximum intensity for such reduced cytochrome bands. They were as follows: a, 604.4 mμ; b, 566.5 mμ; c, 550.2 mμ; and the β band of the three components, 521.0 mμ. Keilin concluded that all the cytochromes have a characteristic absorption spectrum and a strong Soret or γ band in the near ultraviolet.

Amounts of cytochromes present were first measured by Keilin (1925). He gave a relative value for the concentration of cytochrome c among various tissues by noting the depth of the tissue necessary for the microspectroscope to reveal a clear absorption spectrum with four characteristic bands. He stated that a more precise way to determine the relative concentrations of cytochrome c is to compare the thicknesses of compressed tissues at which the c band is extinguished. Through these experiments, it was shown that the concentration of cytochrome c varied not only with the species examined but also, and to a much greater extent, in different tissues of the same animal.

Keilin (1930) obtained evidence that cytochrome b was closest to the substrate side of the chain. He also gave the name "cytochrome oxidase" to a CO-, KCN-, and H_2S- sensitive entity containing Cu which linked the cytochromes to O_2.

Bensley and Hoerr (1934) attempted to isolate mitochondria from liver tissue by means of differential centrifugation. They

failed in their efforts to produce intact mitochondria due to their failure to use appropriate suspending media and correct centrifugation procedures.

Millikan (1937) worked out instruments and techniques for the recording of small optical density changes in living cells suspensions and tissues. Chance (1954) describing Millikan's research wrote: "He used in very simple form two essential features; (1) a differential colorimeter (following Tyndall's principle) that was responsive only to a change in absorption band of the pigment to be studied; (2) a biological system that could exist in two clearly defined states, and in which the transition from one to the other could be made rapidly and at will. Thus the relative intensities of the two light beams are adjusted initially to give zero response in one state, the biological system is shifted to the other state, and the magnitude of the absorption of the cell pigment is recorded directly."

Keilin and Hartree (1939) showed that cytochrome oxidase was identical with cytochrome $\underline{a}_3$. They further stated that the cytochromes, a linear chain of carriers, have a terminal portion consisting of cytochrome $\underline{c}$ in close association with the oxidase. Therefore, the cytochrome chain is grouped as follows:

$$\text{Substrate} \longrightarrow \underline{b} \longrightarrow \underline{c} \longrightarrow \underline{a} \longrightarrow \underline{a}_3 \longrightarrow O_2$$

Keilin and Hartree (1939) also included a table with positions of the absorption bands for reduced cytochromes.

Notation	Position (mμ)
a α	605
a β	?
a γ	452
a_3 α	600
a_3 β	?
a_3 γ	448
b α	564
b β	530
b γ	432
c α	550
c β	521
c γ	415

The spectroscopy of cell pigments in the visible spectrum as done by MacMunn and Keilin is useful for a rapid identification of the types of cytochrome in various microorganisms. As long as one is studying the α and β bands of cytochromes (650 - 500 mμ), this is the best method. But when attempting to study the 5- to 10- fold stronger γ or reduced pyridine nucleotide bands in the region 440 - 400 mμ, larger light-scattering effects at these shorter wavelengths are superimposed upon the light absorption to give a considerably distorted record.

Stotz (1939) devised a method for quantitative measurement of cytochrome c based on the catalytic function of the cytochrome. The determination was made by comparing the rate of oxygen uptake obtained with an unknown solution to that obtained with a standard cytochrome c solution.

Potter and Dubois (1942) stated that extraneous factors present a constant danger in analyses based on rate measurements. They devised an absolute measurement of the cytochrome c concentration. This measurement involved the use of a spectrophotometer to measure the absorption coefficients for reduced and oxidized cytochrome c. Previously, Potter (1941) had suggested that it should be possible to determine the amount of cytochrome c present. This could be done spectrophotometrically by means of the enzymatic oxidation and subsequent reduction of the compound. He based his conclusion on an experiment in which he obtained 100 percent recovery of added cytochrome c when the reduction was carried out in the presence of 0.001 per cent cyanide added after succinate.

Claude (1946) was able to separate the cytoplasmic material of liver cells into three main fractions: (a) a large granule fraction composed of elements approximately 0.5 to 2.0 microns in diameter, corresponding to the mitochondria and the liver secretory granules; (b) a microsome fraction composed of submicroscopic elements 80 to 150 mμ in diameter corresponding to the chromophilic ground substance of the liver cell; and (c) a supernatant fraction containing the soluble components of the cells.

Schneider (1946) showed through biochemical studies that cytochrome oxidase and succinoxidase were concentrated in the large granule fraction. Hogeboom et al. (1948) isolated mitochondria from the large granule fraction by the use of a hypertonic sucrose solution and differential centrifugation. These isolated mitochondria were

predominately rod shaped and stained with Janus green B (two criteria for mitochondria which had not been met in earlier work, possibly due to the procedure which used water and isotonic saline in place of the sucrose solution).

Kennedy and Lehninger (1948) proved that cytochromes are located in mitochondria.

Schneider and Hogeboom (1950) and Beinert (1951) put forth the theory that small amounts of the cytochromes and cytochrome oxidase found in subcellular fractions other than the mitochondrial fraction probably originated in secondary translocations and absorptions during the preparative cell fractionation.

In finding that Millikan's apparatus was suitable only for measuring hemoglobin and myoglobin in the aerobic and anaerobic muscle states, Chance (1951) used two monochromators and a vibrating mirror to pass light of two selected wavelengths through the sample and then upon a photocell. This method was found to be suitable for measurements of both the sharp bands of the cytochromes in the visible region and the reduced pyridine nucleotides in the ultraviolet region. With the development of this double beam equipment, Chance and his colleagues were able to give a more precise picture of the composition, sequence, and dynamics of the respiratory chain using intact mitochondria.

Chance (1952) reported that the difference spectra obtained on reduction of cytochrome and pyridine nucleotide enzymes were useful for the identification of the various cytochrome components, for the

comparison of the cytochromes of different cellular material, and for the quantitative estimation of the relative and absolute cytochrome content of various systems. Chance made some comparisons based on the height of the cytochrome c peak at approximately 551 mμ to its low point at 541 mμ.

The difference spectra of the respiratory carriers in rat liver mitochondria were recorded by Chance and Williams (1956) at 25°C:

Band	Absorption Maxima mμ
a α	604-5
a γ	450
a_3 α	600
a_3 γ	444-5
b α	562-4
b β	530
b γ	430
c α	550
c β	521
c γ	416

These figures for intact mitochondria agreed with those of Keilin and Hartree (1939) showing that the cytochromes as measured earlier by absorption maxima were the same in the intact mitochondria.

Chance et al. (1959) developed a sensitive microspectrophotometric apparatus capable of recording the absorption spectra of an area 1.5 microns in diameter from intact living cells. With this instrument it was possible to record the spectra of mitochondrial aggregates in the cytoplasm of the living cell. Then the cytochromes could be identified and measured.

Luck (1965) collected mitochondria from the filamentous fungus, Neurospora crassa, in bands after centrifugation in a sucrose gradient. These mitochondria were broken down for cytochrome analysis with a sonifier after addition of sodium deoxycholate. A Cary model 14 MR recording spectrophotometer was used to analyze the cytochromes. The tracings showed an α peak for cytochrome a to a_3 at 609 mμ and α peaks for cytochromes b and c at 560 and 550 mμ, respectively. In addition, there were β peaks at 528 mμ for cytochrome b, and at 520 mμ for cytochrome c.

Instead of collecting N. crassa mitochondria on a sucrose gradient, Bertrand and Pittenger (1969) sedimented them into a pellet by centrifuging at 12,000 x g for 20 minutes. This pellet was resuspended in sucrose plus EDTA solution and sonified. The characteristic absorption maxima noted for the cytochromes of N. crassa were:

a - a_3 α	609 mμ
a - a_3 γ	443 mμ
b α	560 mμ
b β	530 mμ
b γ	428 mμ
c α	550 mμ
c β	520 mμ
c γ	418 mμ

Studies on Cytochrome-Deficient Strains of Fungi

The petite characters in the yeast, Saccharomyces cerevisiae were studied by Ephrussi and Hottinguer (1951). The petite yeasts were

slow in growth, formed small colonies, and had abnormalities in mitochondrial cytochrome systems. The phenotype resulted either from nuclear gene mutation or from alterations in cytoplasmic inheritance. There were two types of petites resulting from alterations in cytoplasmic inheritance. One type yielded only normal yeast among the progeny from a cross to normal, while the other yielded only petites in crosses to normal. Petite yeast lacked the cytochrome a, a_3, and b of wild type yeast but had a greater amount of cytochrome c than wild type (Wagner and Mitchell, 1964).

Mithcell and Mitchell (1952) reported that the slow growth characteristic of a strain of the filamentous fungus, Neurospora crassa, was maternally inherited. They showed that in order for this characteristic to appear in all of the progeny, it must be carried by the protoperithecial parent, which furnished the greater part of the cytoplasm for the ascospores. Mitchell and Mitchell called this strain "poky" (now identified as maternally-inherited -1 or [mi-1]). The [mi-1] character resembled the petite character in yeast described by Ephrussi and Hottinguer (1951). There was a difficulty in making a simple comparison of the two, since the yeast ascospores arose from a mixture of the cytoplasm of the two parents, whereas in [mi-1] it appeared probable that such mixing did not occur. The growth rate of [mi-1]strains was greatly reduced from that of the wild type and similar to that of biochemical mutants on minimal medium. The strain [mi-1] did not assume a normal growth rate on any type of enriched medium.

Haskins *et al.* (1953) reported mycelial pads from [*mi-1*] were characterized by a red color similar to that of the heme pigments. Examination of these mycelial pads with a spectroscope showed that [*mi-1*] mycelia contained large quantities of a substance having the absorption spectrum characteristics of cytochrome *c*, whereas cytochrome *a* and *b* bands could not be detected. In contrast, all three cytochromes were detected in wild type.

Mitchell *et al.* (1953) reported that [*mi-1*] contained 15 times as much cytochrome *c* as wild type, based on the width of the absorption band of cytochrome *c* in [*mi-1*]as compared to that in wild type *Neurospora crassa*.

Woodward and Munkres (1966) found that the mitochondrial structural protein from [*mi-1*] had one less tryptophan residue per mole of mitochondrial structural protein and one more cysteine residue than did wild type mitochondrial structural protein. Thus, the mutation appeared to result from a single amino acid replacement resulting from an alteration in the mitochondrial DNA. A pleiotropic effect based on nucleocytoplasmic interactions seemed to be the cause of the slow growth accompanied by a large amount of cytochrome *c* and absence of cytochromes *a*, a_3, and *b*, with cytochrome oxidase and succinic acid oxidase having lower activities than in the wild type.

Heller and Smith (1966) reported that cytochrome *c* of *Neurospora crassa* wild type and [*mi-1*] were structurally identical. The experimental proof consisted of identical spectral properties,

electrophoretic mobility at different pH values, amino acid composition and peptide maps from the chymotryptic and tryptic digests.

The [mi-1] type strain used in this study was produced by the micro-injection of mitochondria from an [mi-1] strain of Neurospora crassa into a wild type strain. After several serial transfers, this wild type strain developed the [mi-1] phenotype (Wilson, unpublished).

Wilson (1961) developed surgical techniques specifically for use with Neurospora crassa. With these techniques, he could perform 60-80 injections in one or two hours.

Diacumakos et al. (1965) described the drastic changes in the rate of growth, morphology, reproductive characteristics and cytochrome spectra of a normal inositol-less strain of Neurospora crassa (FGSC #948) after micro-injection with mitochondrial preparations from an abnormal inositol-less strain (FGSC #1448). Abnormal 1 ([Abn-1]) was originally described by Garnjobst et al. (1965). No change was noted in a wild type strain micro-injected with wild type mitochondria. Diacumakos reported that there was a considerable and variable time lapse between injection and appearance of the characteristic changes in morphology and growth rate.

Griffiths et al. (1968) repeated the work of Mitchell et al. (1953) and Tissieres and Mitchell (1954), but derived the cytochrome spectra from disrupted mitochondrial preparations instead of from the mycelial pads and crude mitochondrial suspensions used by the two earlier research groups. Mitochondria were prepared by a method similar to that used by Luck (1965). Mitochondria were sedimented in

a 30 minute centrifugation at 20,000 x g. The crude mitochondrial pellets were disrupted by sonification and the solutions cleared by addition of 2 per cent sodium deoxycholate. Spectra were read in a Cary 16 spectrophotometer, with sodium dithionite added to the sample cuvette to reduce the cytochromes. The solutions for spectra analysis contained 10-20 mg/ml of protein estimated by Folin test. The cytoplasmic mutants they scanned fell into two groups based on their spectra. The first group had [mi-1] spectra with the notable features being an absence of cytochromes a (610 mμ) and b (560 mμ), and a marked ∝ cytochrome c peak (550 mμ). The second group consisting of [mi-2] to [mi-8] was missing cytochrome a, while cytochrome b was present in wild type amounts and cytochrome c was in excess.

Wilson (unpublished) has shown that the [mi-1] phenotype can be transmitted to wild type Neurospora crassa by micro-injection (or transplantation of [mi-1] mitochondria). The author of this study followed this change in phenotype by changes in the cytochrome spectra and quantitated these changes. These changes were followed using a Beckman DB Spectrophotometer to measure the amount of cytochrome c per unit weight of protein and to record the distribution of cytochromes at each stage.

CHAPTER II

METHODS AND MATERIALS

The strains used in these studies are listed in Table I. All strains were obtained from Dr. J. F. Wilson, Biology Department, University of North Carolina at Greensboro.

Dr. Wilson obtained NC-37401-NS-10 (2-6) a inos (abbreviated NS-10) from crossing 37401- F_3 -1 (4-5) a inos (abbreviated 37401), the conidial parent, with NS#8 a inos [mi-1] f (abbreviated NS #8), the protoperithecial parent. He micro-injected [mi-1] mitochondria from SL-3 (1-6) a nic-2 [mi-1] (abbreviated SL-3) into HC (23-8) a (abbreviated HC), creating strain P-35-6. Strain P-35-1C is a control produced by micro-injection of wild type HC mitochondria into HC cells.

The strains used in this experiment were first grown on slants of Vogel's (1956) minimal medium supplemented with 1.5 per cent agar, (General Biochemical) and 1.5 per cent sucrose (Fisher). In the case of inositol-less or nicotinamide-less strains 50 mg/ml inositol (Sigma) or 1 mg/ml nicotinamide (Niacinamide Hydrochloride, Nutritional Biochemicals) was added. The slants were incubated at 30°C for three days. Within two to five days after removal from the incubator, conidia which formed on the slants were transferred to six or eight flasks of the same composition. After the same incubation

TABLE 1

SOME STRAINS USED IN THESE STUDIES

Culture	Mating Type	Heterocaryon Compatibility Genotype
37401-F -1 (4-5) *inos*	A	CDE
NC-OR-66 (2-7)	a	Cde
NS#8 *inos* [*mi-1*] *f*	a	CdE
NC-37401-NS-10 (2-6) *inos*	a	CDE
P-35-6	a	CDe
P-35-1C	a	CDe
HC (23-8)	a	CDe
SL-3 (1-6) *nic-2* [*mi-1*]	a	Cde

and conidial formation period as the slants, the conidia were harvested from the flasks by washing with sterile water. The mycelia were strained out by a triple layer of sterile gauze, allowing the conidia to drop through into a flask. Conidial counts were done on a hemacytometer. A conidial count of 1×10^6 conidia/ml for wild type and 5×10^6 conidia/ml for [mi-1] was applied to a liquid medium of 150 ml of Vogel's minimal media supplemented with 2 percent sucrose. Twelve inoculated flasks for wild type and either 18 or 20 inoculated flasks for [mi-1] were incubated on a rotary shaker (Brunswick Psycotherm Environmental Incubator Shaker) for 16 hours at 150 rpm and 25°C.

The mycelia were harvested on filter paper in a Buchner funnel. They were prepared for cytochrome analysis by a slightly modified method of Luck's (1965). The method of preparation is:

1. Rinse mycelia mat with solution of 15 percent sucrose + 1 mM EDTA.
2. Grind 1:1 with acid-washed sand (Omaha sand, Fisher), about 1:1 with 15 percent sucrose + 1 mM EDTA. A cell disruption bomb (Parr #4635) was tried in an attempt to find a more successful way to disrupt the cells, but the effort proved unsuccessful.
3. Centrifuge 15 minutes in Sorvall Superspeed RC 2-B at 500 rpm (30 x g).
4. Centrifuge supernate 15 minutes in Sorvall at 1000 rpm (121 x g).
5. Centrifuge supernate 20 minutes in Sorvall at 11,500 rpm (15,900 x g).
6. Resuspend pellet in 7 ml of 0.15 M PO_4 buffer pH = 7.4.

7. Centrifuge solution 15 minutes in Sorvall at 1000 rpm (121 x g).

8. Sonify supernate on Bronson Sonifier according to the following procedure: 5 seconds at #5 reading, 30 seconds shaking in ice solution, 5 seconds at #6 reading, 30 seconds shaking in ice solution, 6 seconds at #6 reading, 30 seconds shaking in ice solution, 6 seconds at #6 reading, followed by placing mitochondrial solution on ice. Extreme care should be taken to keep the solution cold throughout the procedure.

After the above procedure, the mitochondria were ready for cytochrome analysis. The instrument used for this analysis was a Beckman Model DB Spectrophotometer used with a Beckman Scale Expander, with the results recorded on a Beckman Linear-Log Recorder. The quantitative analysis was based on the fact that the absorbances of cytochromes were dependent on their concentrations (Beer's Law). Beer's Law states: $A = abc$ where A is the absorbance; a is the absorbtivity (unit area per unit mass), b is the length of the light path, and c is the concentration (mass per unit volume).

Since a matched set of cuvettes with a light path of 1 cm and material with the same absorption (Neurospora crassa mitochondria brought up to volume of 1.5 ml by the addition of 0.15M PO_4 buffer pH = 7.4) were used, the variable of Beer's Law on which the absorbance depended was the concentration of cytochrome present.

A solution of mitochondria prepared from mycelia by Luck's method previously described was placed in cuvette and brought to 1.50 ml of volume. Then 0.15 ml of a 2 percent solution of sodium deoxycholate (Difco) was added to dissolve the mitochondrial membrane and clear some of the remaining lipids in the cytochrome

solution. A second cuvette was prepared in the same manner.

These cuvettes were placed in the Beckman DB (double beam) Spectrophotometer, one in the reference beam and one in the sample beam. The slit adjustment was turned to a programmed narrow slit with the manual slit adjustment in as far as possible. The spectrophotometer was set to scan the sample from 700 mμ moving at the rate of 40 mμ per minute and to record the difference between the reference cuvette and the sample cuvette. The ten-inch recorder was set for 10 millivolts for full scale deflection, with the chart driven at one inch per minute with logarithmic presentation.

Quantitative analysis of cytochromes in sonified mitochondrial preparations required detection of small absorbancies in the presence of highly turbid, and therefore highly absorbing, samples. An instrument with the required sensitivity at high absorbancies was not available for these studies; so a method developed by Dr. William K. Bates for the Beckman Model DB Spectrophotometer was used. This method took advantage of the relatively high signal to noise characteristic of the DB spectrophotometer by using relatively dilute preparations, which contributed proportionally small amounts of turbidity, and then amplified the corresponding low cytochrome absorbance. This was accomplished by interposing a Beckman Scale expander between the spectrophotometer and the recorder and using the expander to attenuate the output signal. The usual mv spectrophotometer output was reduced through the "expander" so that a 10 mv recorder sensitivity could be used. With the recorder used

in the logarithmic mode, the resulting trace was then expanded according to the setting of the scale expander, but with the disadvantage that the resulting trace was linear in neither percent transmittance nor absorbance. Quantitation, therefore, was possible only through direct comparison at many calibration points with a cytochrome standard. Operationally, with the scale expander set to "5" and with the recorder operated on the 10 mv scale, a suitably amplified trace resulted. The "bucking" circuit of the expander was used to set the base line of the trace at a suitable starting point on the tracing paper. It should be noted that the setting of "5" would yield a full-scale deflection for 20 percent transmittance if the recorder were operated in the linear mode with 100 mv full-scale sensitivity.

The mitochondrial preparations were first scanned in the oxidized state by scanning the cuvettes containing the mitochondrial solutions, 0.15M PO_4 buffer pH = 7.4, and sodium deoxycholate and adjusting the starting absorbancy so the oxidized state line was recorded at the height of the number 10 line on the recording paper. This line formed by the absorption of light by the oxidized cytochromes was used by the author to find any peaks present not used by reduction in order to subtract their height from the peak height of the reduced cytochromes and also to show the presence of any turbidity which would cause the line to rise as the scan proceeded from 700 to 400 mμ.

After the oxidized cytochromes were scanned, the paper was returned to the starting point of the oxidized scan. A small amount

of sodium dithionite (Fisher) was added to the solution in the sample cuvette to reduce the cytochromes. Then the cytochromes were rescanned with the starting absorption line adjusted to a position between line 15 and line 24. A trial was run measuring the peak height of the cytochrome c peak for 0.7 mg sample of strain P-35-6 transfer 10 as a function of various base lines. The peak height remained 12 millimeters over base lines ranging from 14.5 to 24.

Table 2 lists the positions where cytochrome absorbancy peaks were detected.

The mitochondrial suspension was kept in an ice bath until it was placed in the cuvettes. As the cytochrome solution was used in the cuvettes it gradually became turbid, but by working rapidly the scans could be made before the turbidity affected the results. The presence of the buffer kept a change in pH from occurring, which would have thrown off the relationship between the light absorption and the cytochrome concentration.

The peaks produced by the absorption of light were measured in a method modified from that of Chance (1952). Chance measured the height of the cytochrome c peak from its high point at approximately 551 mμ to its low point at 541 mμ. The author of this present study modified Chance's method by drawing a line horizontally between the low points for wild type 588 mμ or [mi-1] 550 mμ and 532 mμ. Then the peak height was measured from the almost horizontal line between the low points and the peak height. This method had a built-in

TABLE 2

POSITIONS OF CYTOCHROME ABSORBANCY PEAKS

Cytochromes	mμ
$\underline{a}$-$\underline{a}_3$ α	609
$\underline{a}$-$\underline{a}_3$ γ	445
$\underline{b}$ α	560
$\underline{b}$ β	528
$\underline{b}$ γ	428
$\underline{c}$ α	550
$\underline{c}$ β	520
$\underline{c}$ γ	418

compensation for the rise due to turbidity which was forming in the solution and causing the base line to rise. The measurement of the peak height formed a better correlation with the amount of cytochrome c present than the measurement of the area beneath the curve. For example, when the protein concentration doubled, the peak height doubled.

The protein concentration of each sample was determined by Biuret method (Gornall et al. 1949). With this information, the amount of protein required to produce a certain size cytochrome c peak could be determined for each strain.

In order to correlate the height of the peaks with a given amount of cytochrome c, samples of cytochrome c of known weight and purity were scanned and measured. A source of 90 percent pure cytochrome c was available in the form of horse heart cytochrome c (Nutritional Biochemicals Corporation, Lot #9478). Literature from Nutritional Biochemicals Corporation listed reduced horse heart cytochrome c as demonstrating absorption bands at 550.7 mμ, 522.3 mμ, and 415.0 mμ. Cytochrome c from Neurospora crassa can be compared to that of horse heart cytochrome c due to the fact that the cytochrome c is very similar in all organisms. Those differences which have arisen have been slight (Dickerson and Geis, 1969).

A cytochrome c solution was prepared by dissolving 0.0042 g of the horse heart cytochrome c in 100 ml of 0.15 M PO_4 buffer pH = 7.4. Then 0.1 ml increments of cytochrome c solution were placed in cuvettes

and raised to a volume of 1.5 ml by the addition of 0.15 PO_4 buffer pH buffer = 7.4. The sample was ready to scan after it was cleared with 0.15 ml of 2 percent sodium deoxycholate. The mixture was scanned in the oxidized state, a few crystals of sodium dithionite were added, and it was scanned again to record the reduced cytochrome c peaks.

By determining the amount of cytochrome c which produced a certain size c peak, strains were compared to each other and the amount of cytochrome c per mg of protein determined for each.

Comparative studies involving [mi-1]and wild type mitochondria were well suited for quantitative analysis based on the absorption spectra of the cytochromes.

The first organism to which the quantitative analysis was applied was NS#10. This strain had a wild type amount of cytochrome c; it lacked cytochrome a, a_3, and b; and it possessed a [mi-1] type growth rate. Logically, the cross of NS#8 (protoperithecial parent) with 37401 (conidial parent) would produce the cytochrome pattern and quantity of cytochrome c present to match that of NS#8, for mitochondria are maternally inherited. The first approach to a study of this problem was to grow and scan the NS-10, NS#8, and 37401 and to obtain the cytochrome c peak height as a function of the protein concentration for each. Then, mitochondria could be micro-injected into wild type strains in different amounts. Next, the micro-injected strains could be scanned and plotted. The data would be compared to those of the scans of NS-10, NS#8 and 37401.

Another problem which appeared to lend itself to analysis by the quantitation method was the study of the P-35-6 series ([mi-1] mitochondria injected into wild type cells).

P-35-6 and a control P-35-1C were obtained in sets of the original up to transfer 15 from Dr. J. F. Wilson. He had transferred the original conidia to a new slant. When the new transplant produced conidia, he transferred it again. This process continued until he had a set of slants for P-35-6 and P-35-1C which contained the original through transfer 15. The author of this study then transferred these sets to slants, to agar flasks and to liquid media flasks. Therefore, the sets that contained the original to transfer 15 (by the time they were ready for scanning) were actually transfers 3 to 18. The steps by which micro-injected mitochondria changed the phenotype of the wild type cell were studied by observing the cytochrome pattern and measuring the cytochrome c per unit weight of protein present.

CHAPTER III

RESULTS

Sodium Dithionite Addition

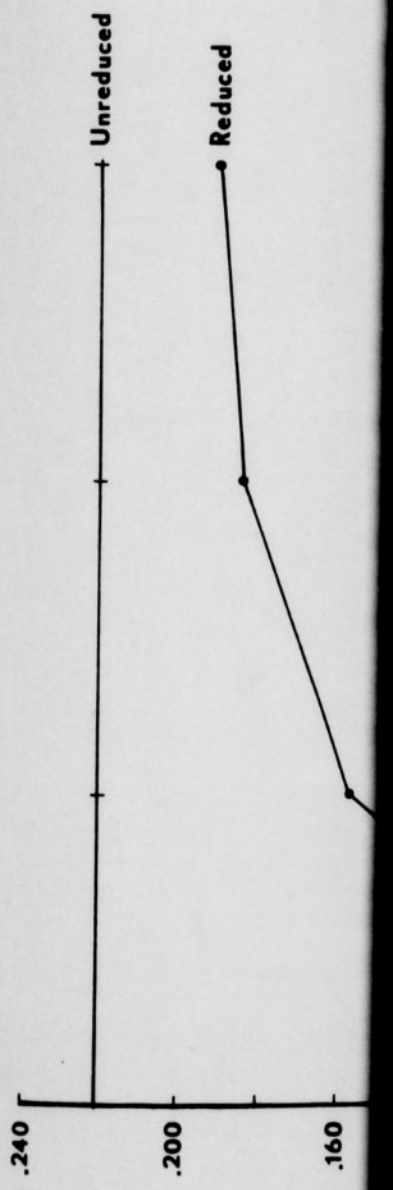
An attempt was made to place sodium dithionite in solution so that a certain number of ml of the solution could be added to the sample cell to cause reduction. Methylene blue dye (Bactomethylene Blue, Difco) which changes color from a blue oxidized to a colorless reduced form was added to the sodium dithionite. Figure 1 shows that 0.1 ml of a 0.0234 g/ml solution of sodium dithionite (approximately the same amount applied in dry form to the cuvette) added to 1.5 ml of 0.002 percent methylene blue caused the absorption at 668 mμ to be 0.064 as compared to 0.220 for unreduced methylene blue. When the same amount of sodium dithionite solution (after standing one hour) was added to a new cuvette of methylene blue, the absorption increased to 0.156. After two hours the absorption was 0.184 and after three hours, 0.189. Therefore, sodium dithionite at the approximate concentration to reduce the cytochromes proved too unstable to keep in solution form.

Cytochrome *c* Standardization

The scan for horse heart cytochrome *c* solution mentioned in a previous section is shown in Figure 2. This scan showed that the

Figure 1

STABILITY OF SODIUM DITHIONITE IN SOLUTION



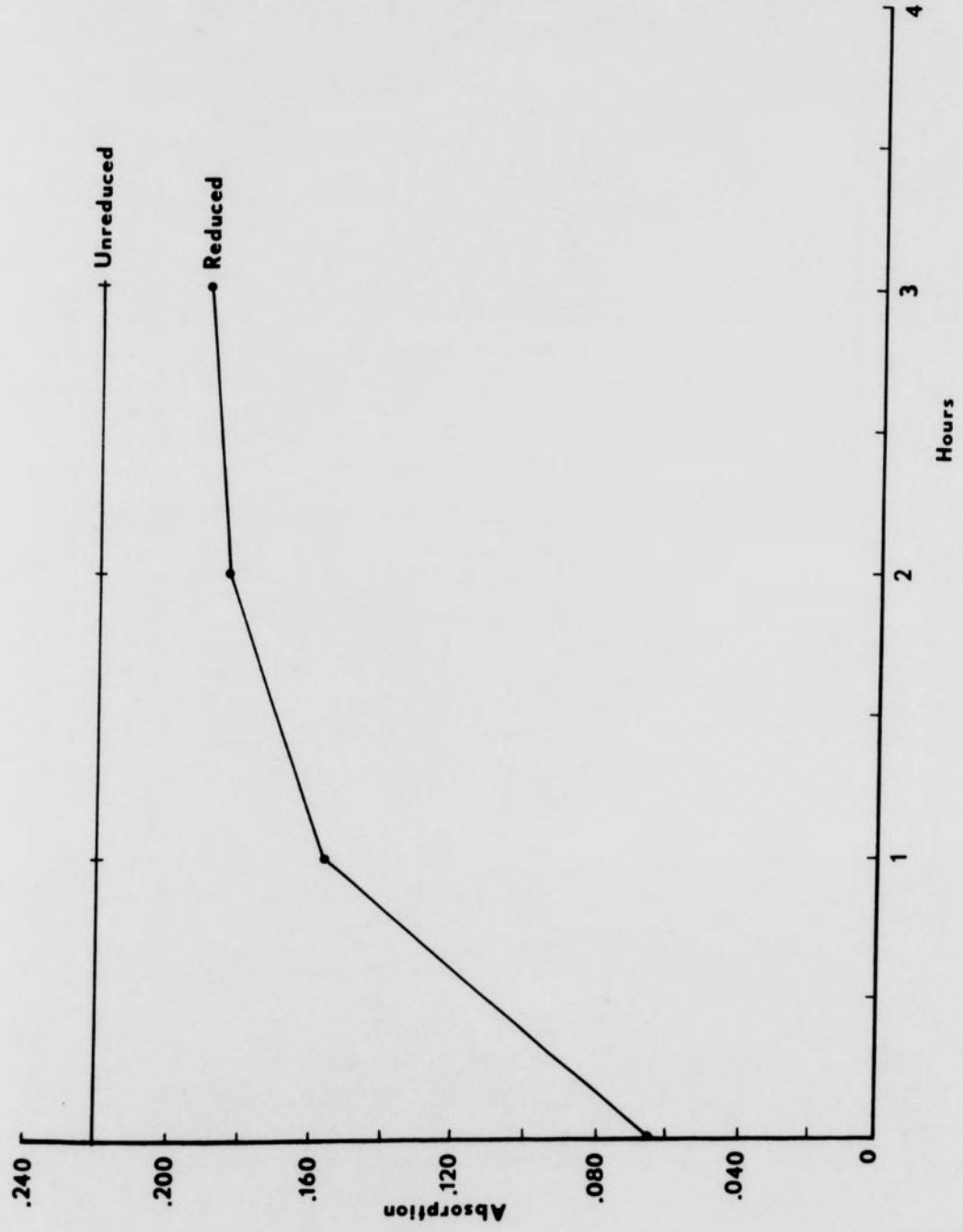
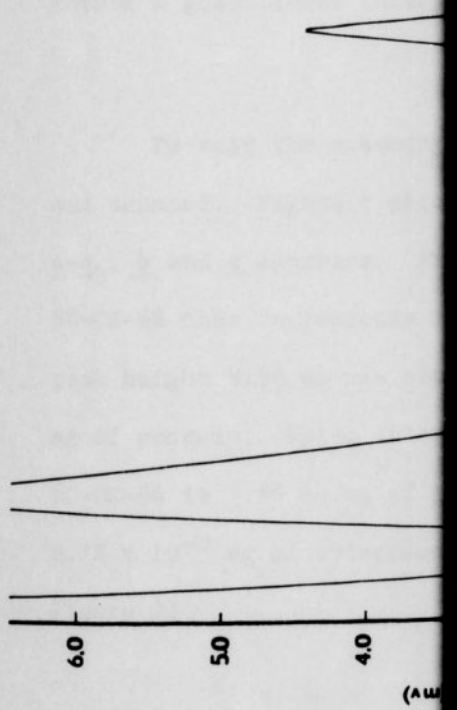
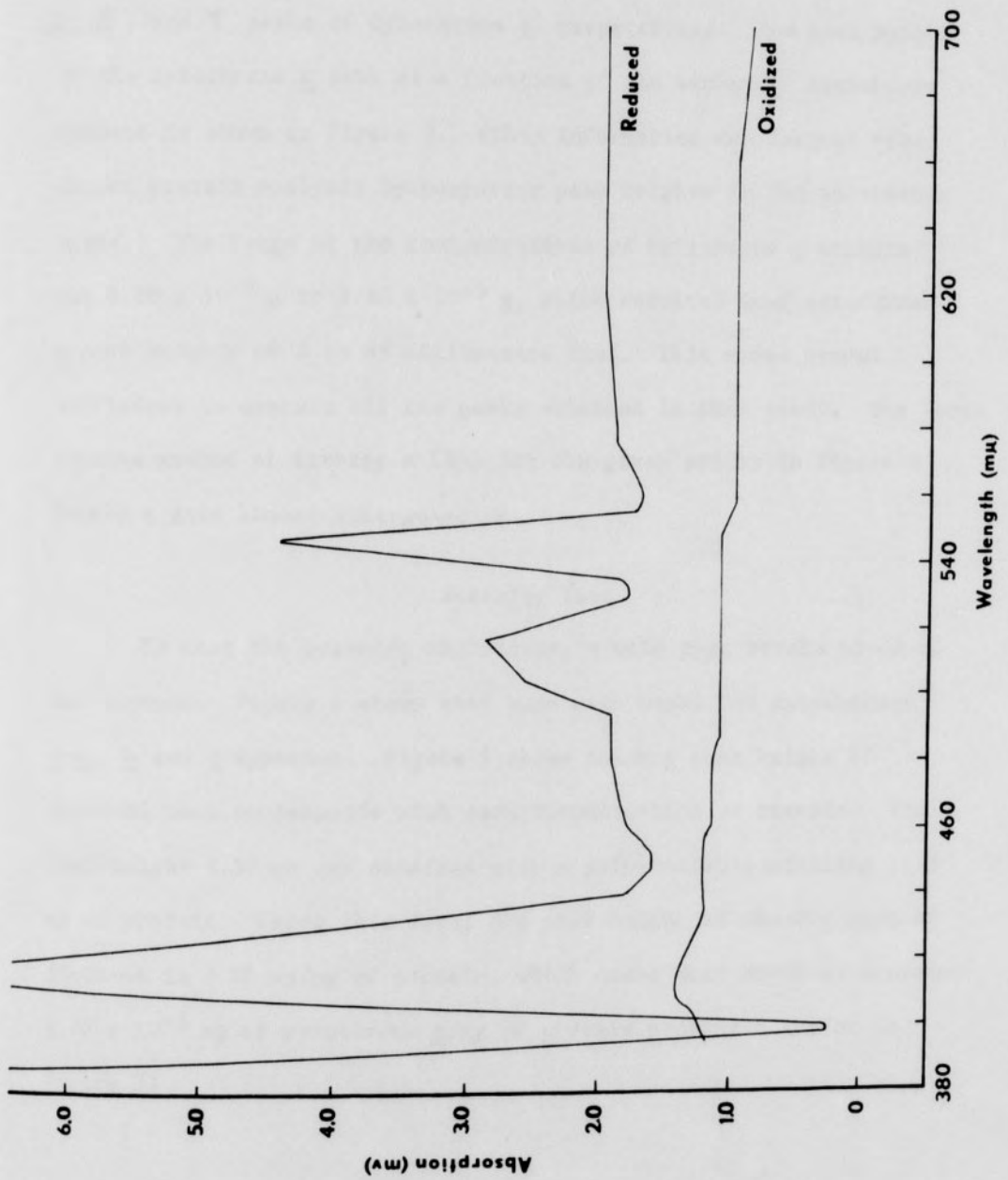

Unreduced
Reduced
.240
.200
.160
.120
.080
.040
0
Absorption
1
2
3
4
Hours

Figure 2

CYTOCHROME ABSORPTION PATTERN HORSEHEART CYTOCHROME C




Reduced
Oxidized
Absorption (mv)
6.0
5.0
4.0
3.0
2.0
1.0
0
380
460
540
620
700
Wavelength (mμ)

cytochrome c solution yielded three peaks with their maximum height at 550 mμ, 520 mμ, and 416 mμ. These three peaks corresponded to the α, β, and γ peaks of cytochrome c respectively. The peak height of the cytochrome c peak as a function of the amount of cytochrome present is shown in Figure 3. (This information was derived from Biuret protein analysis by comparing peak heights in the successive scans.) The range of the concentrations of cytochrome c measured was 3.80×10^{-6} g to 3.80×10^{-5} g, which resulted in α cytochrome c peak heights of 5 to 65 millimeters (mm). This range proved sufficient to contain all the peaks obtained in this study. The least squares method of fitting a line for the given points in Figure 3 formed a good linear interpolation.

Scanning Test

To test the scanning techniques, a wild type strain NC-OR-66 was scanned. Figure 4 shows that scan with peaks for cytochromes a-a_3, b and c apparent. Figure 5 shows the α c peak height of NC-OR-66 that corresponds with each concentration of protein. The peak height 9.50 mm was obtained with a preparation containing 1.25 mg of protein. Using this data, the peak height of the α c peak of NC-OR-66 is 7.60 mm/mg of protein, which means that NC-OR-66 contains 6.78×10^{-3} mg of cytochrome c/mg of protein present. (Refer to Figure 3)

Figure 3

PEAK HEIGHT AS A FUNCTION OF CYTOCHROME C CONCENTRATION
HORSEHEART CYTOCHROME C

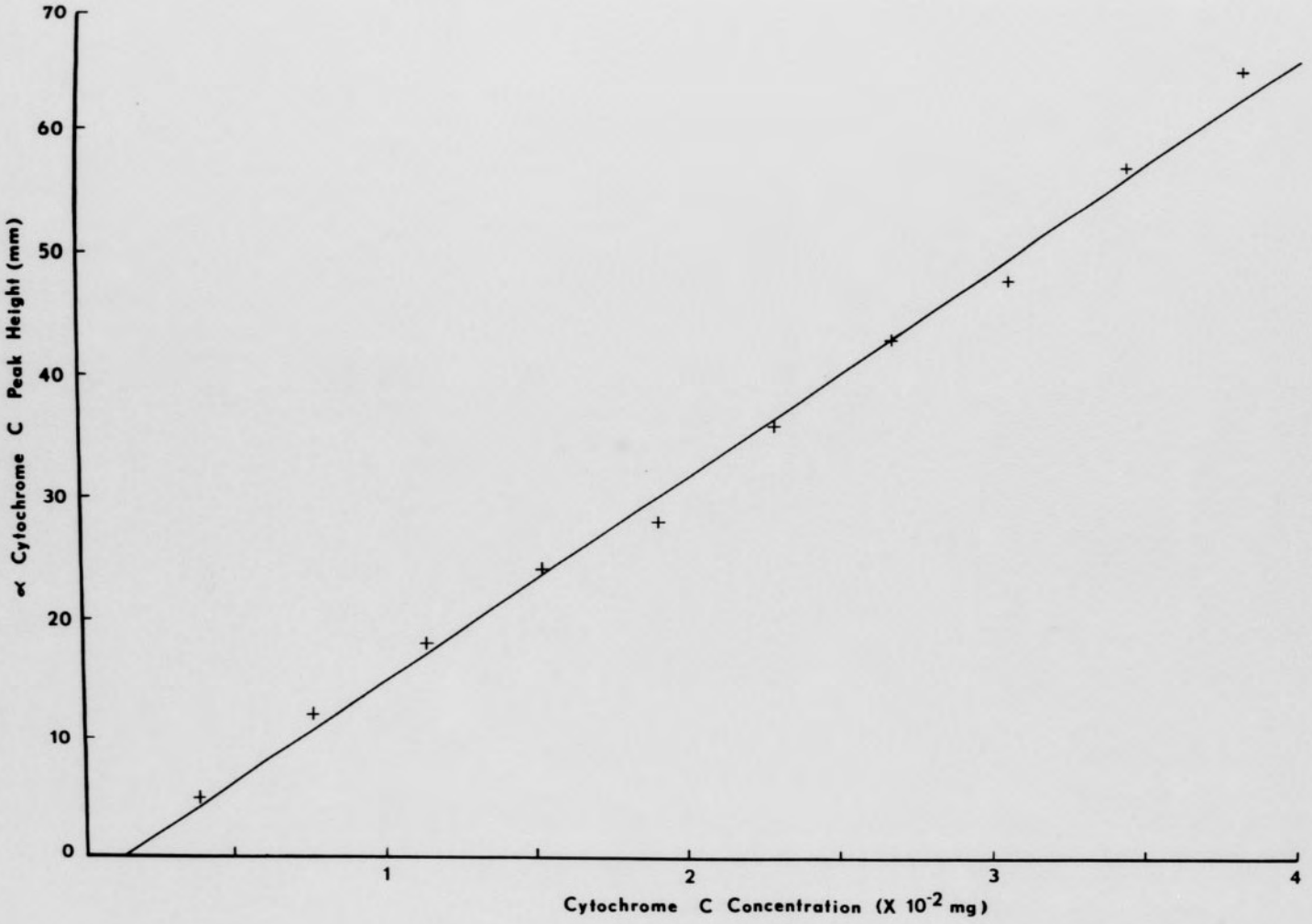
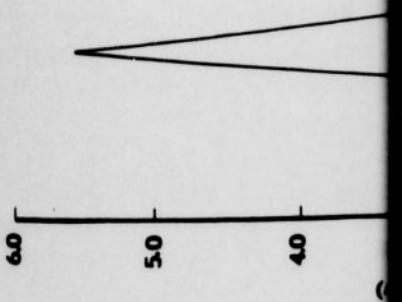

70
60
50
40
30
20
10
0
α Cytochrome C Peak Height (mm)
1
2
3
4
Cytochrome C Concentration (X 10-2 mg)

Figure 4

CYTOCHROME ABSORPTION PATTERN NC-OR-66



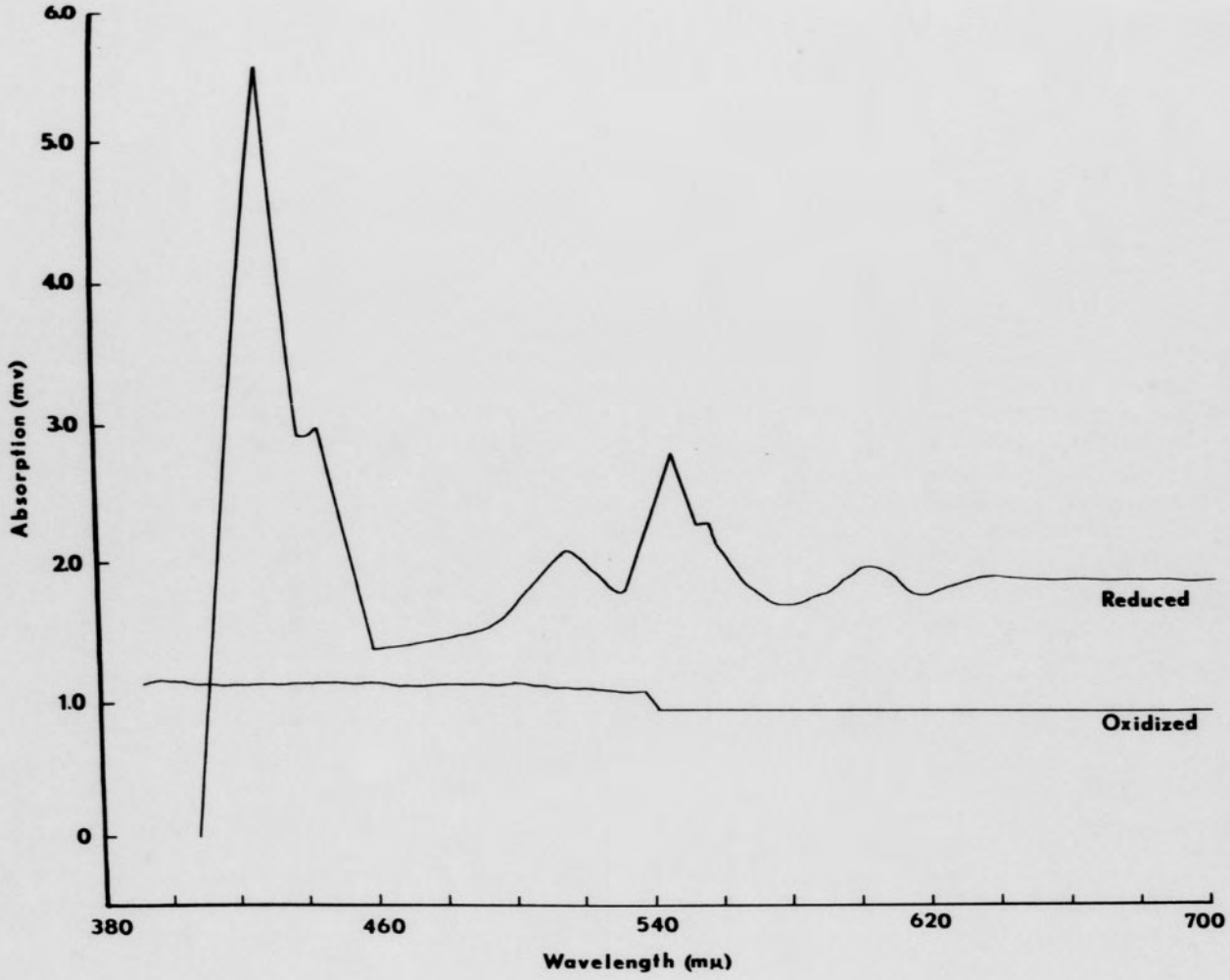
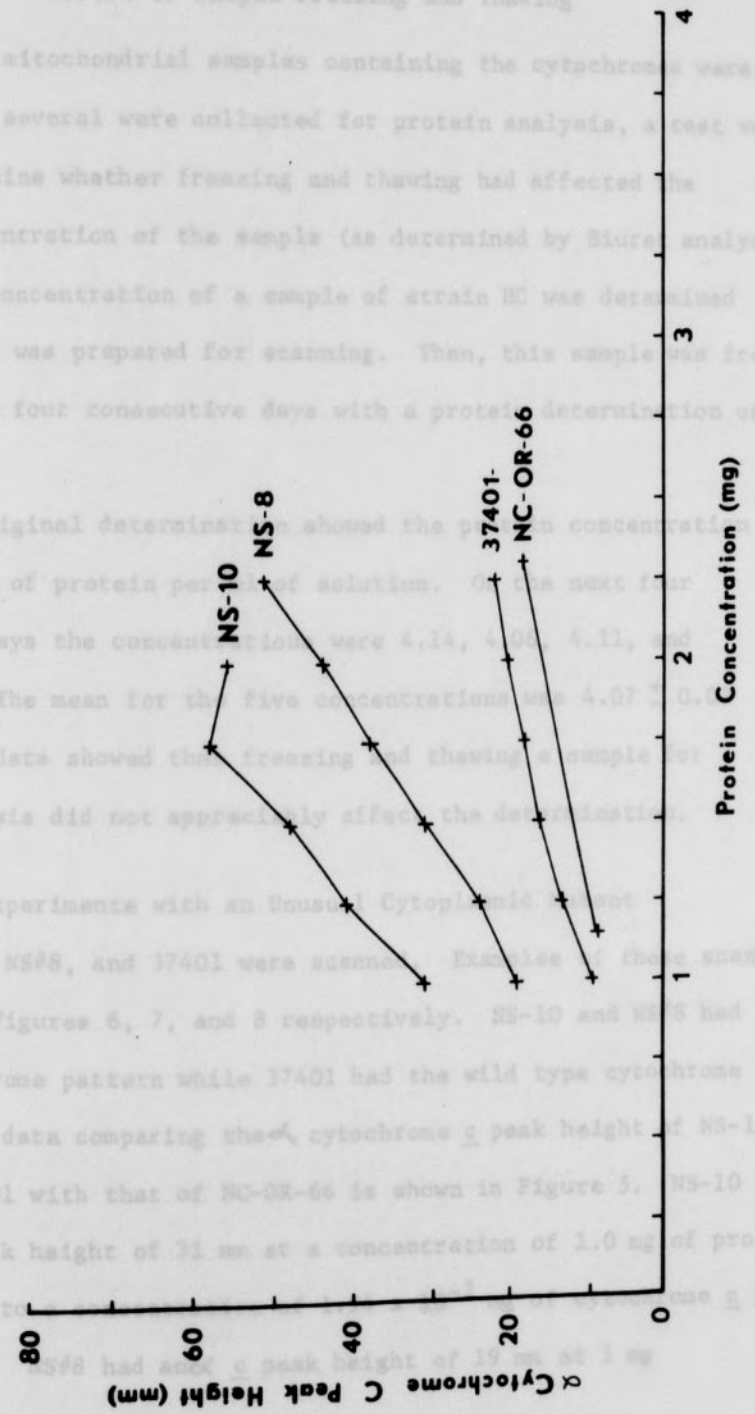

6.0
5.0
4.0
3.0
2.0
1.0
0
Absorption (mv)
Reduced
Oxidized
380
460
540
620
700
Wavelength (mμ)

Figure 5

α_c PEAK HEIGHT AS A FUNCTION OF CYTOCHROME C CONCENTRATION
NS-10, NS#8, 37401 and NC-OR-66


α Cytochrome C Peak Height (mm)
80
60
40
20
0
1
2
3
4
Protein Concentration (mg)
NS-10
NS-8
37401-
NC-OR-66

Effect of Sample Freezing and Thawing

Since mitochondrial samples containing the cytochromes were frozen until several were collected for protein analysis, a test was run to determine whether freezing and thawing had affected the protein concentration of the sample (as determined by Biuret analysis). The protein concentration of a sample of strain HC was determined as soon as it was prepared for scanning. Then, this sample was frozen and thawed on four consecutive days with a protein determination on each day.

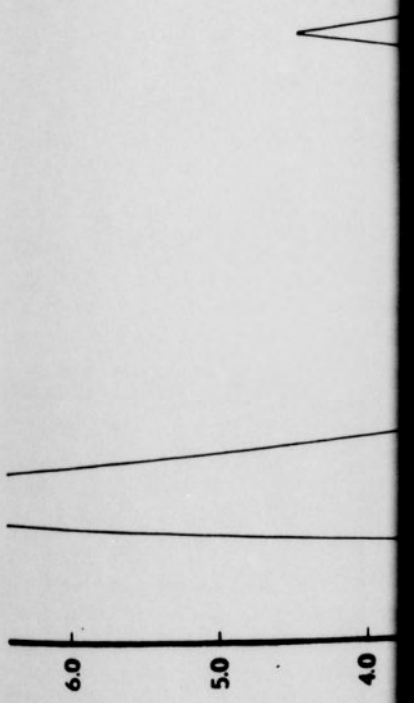
The original determination showed the protein concentration to be 3.98 mg of protein per ml of solution. On the next four consecutive days the concentrations were 4.14, 4.06, 4.11, and 4.00 mg/ml. The mean for the five concentrations was 4.07 ± 0.02 mg/ml. This data showed that freezing and thawing a sample for protein analysis did not appreciably affect the determination.

Experiments with an Unusual Cytoplasmic Mutant

NS-10, NS#8, and 37401 were scanned. Examples of these scans are shown in Figures 6, 7, and 8 respectively. NS-10 and NS#8 had the [mi-1] cytochrome pattern while 37401 had the wild type cytochrome pattern. The data comparing the α cytochrome c peak height of NS-10, NS#8, and 37401 with that of NC-OR-66 is shown in Figure 5. NS-10 had an α c peak height of 31 mm at a concentration of 1.0 mg of protein corresponding to a concentration of 1.94×10^{-2} mg of cytochrome c per mg of protein. NS#8 had an α c peak height of 19 mm at 1 mg

Figure 6

CYTOCHROME ABSORPTION PATTERN NS-10



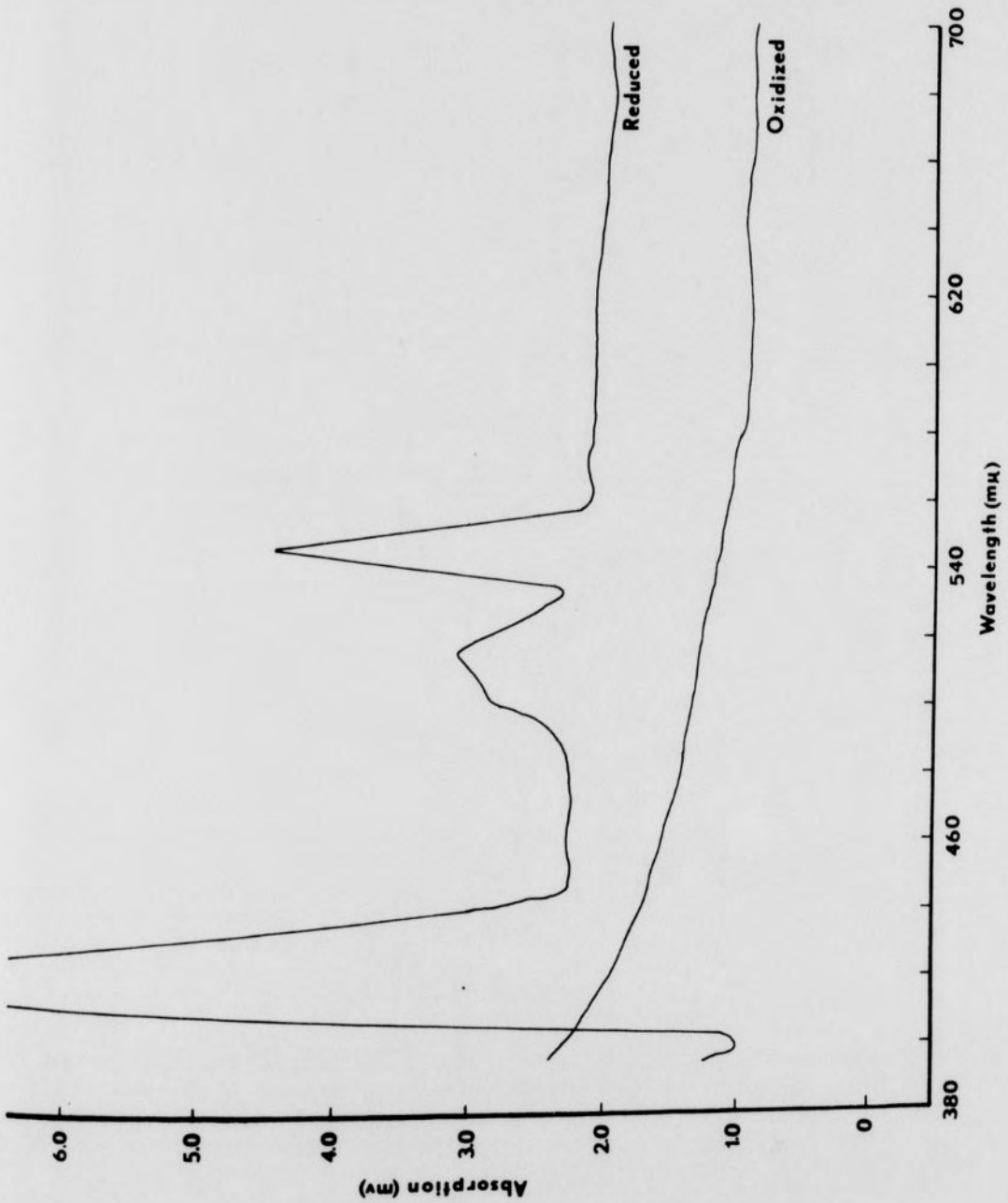
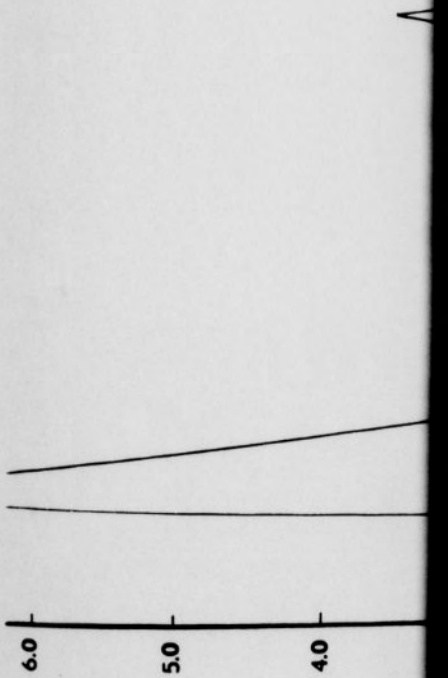

Absorption (mv)
6.0
5.0
4.0
3.0
2.0
1.0
0
380
460
540
620
700
Wavelength (mμ)
Reduced
Oxidized

Figure 7

CYTOCHROME ABSORPTION PATTERN NS#8



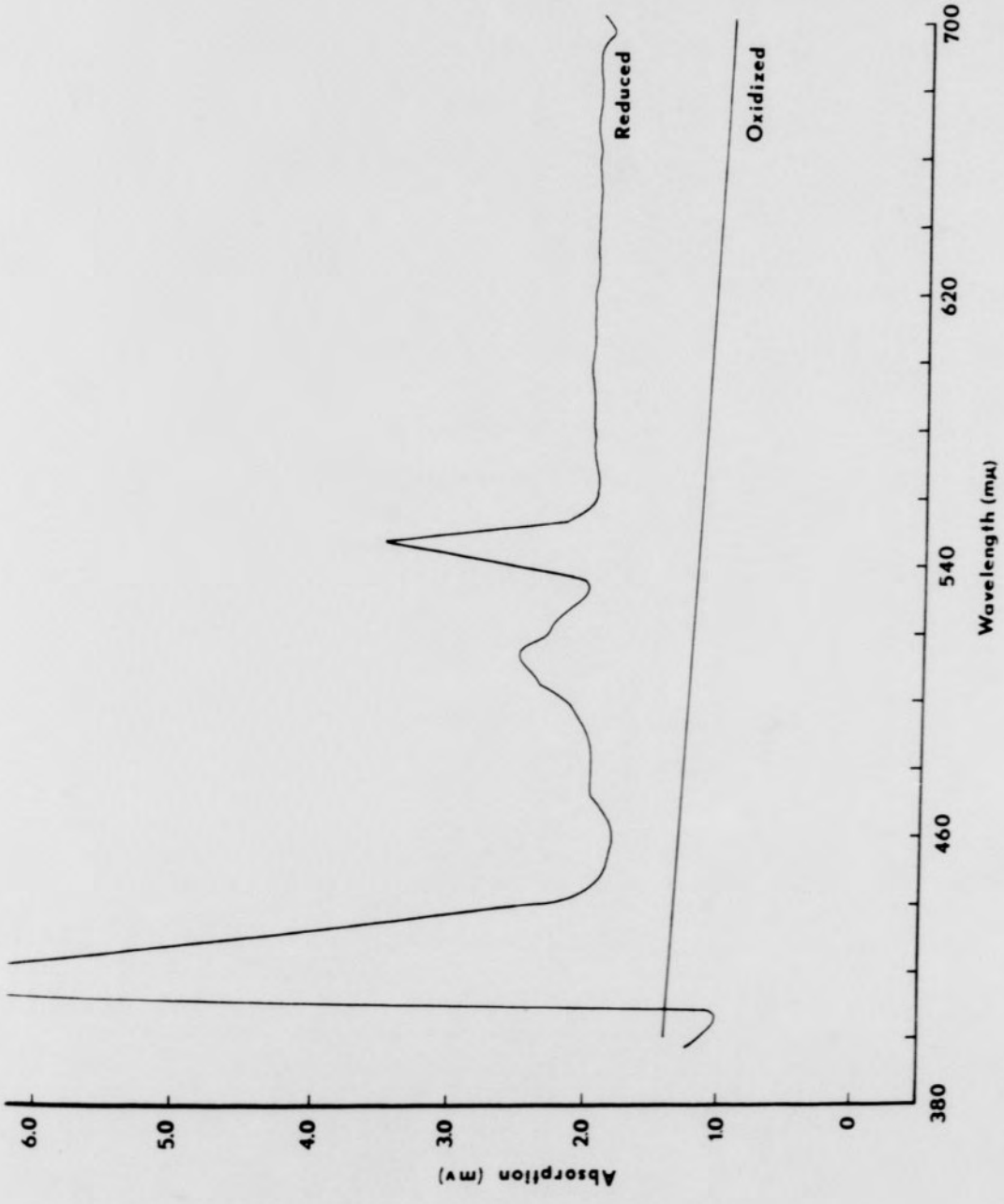
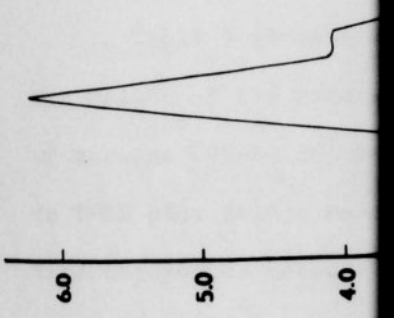
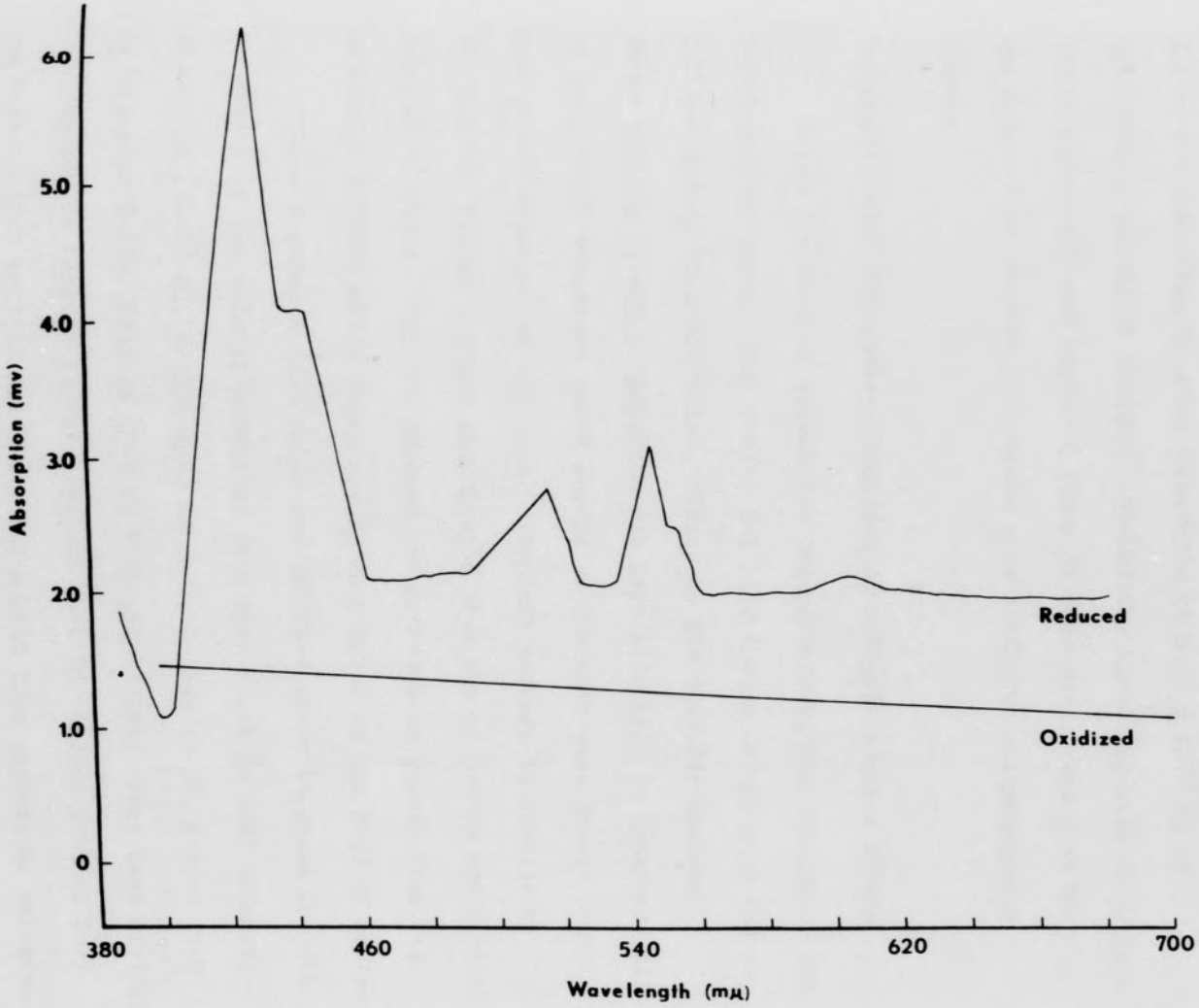

Absorption (mv)
6.0
5.0
4.0
3.0
2.0
1.0
0
380
460
540
620
700
Wavelength (mμ)
Reduced
Oxidized

Figure 8

CYTOCHROME ABSORPTION PATTERN 37401




6.0
5.0
4.0
3.0
2.0
1.0
0
Absorption (mv)
Reduced
Oxidized
380
460
540
620
700
Wavelength (mμ)

corresponding to a concentration of 1.23×10^{-2} mg of cytochrome c per mg of protein. Strain 37401 had an ∝ c peak height of 9 mm at 1.0 mg corresponding to a concentration of 6.48×10^{-3} mg of cytochrome c per mg of protein. Therefore, NS-10 appeared to contain approximately one and one-half times as much cytochrome c as NS#8 and three times as much cytochrome c as 37401 per unit weight of protein.

Experiments with Successive Transfers of a Micro-Injected Strain

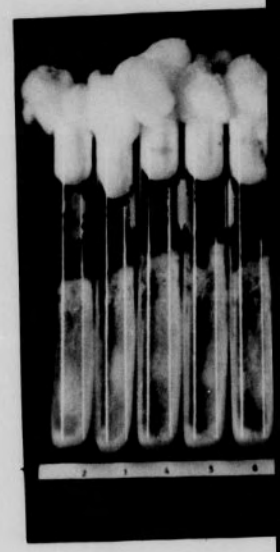
After two days of growth for the transfers, the results of the P-35-6 studies showed that P-35-6 T-1 to T-7 were orange with T-8 to T-16 decreasing in conidiation. Thus, as the transfer numbers became larger, growth of mycelium was less evident. In contrast, all of the P-35-1C transfers which served as controls were orange with good growth apparent at two days (typical pattern in studies by Dr. Wilson). Figure 9 shows photographs of a set of P-35-6 and P-35-1C taken at 48 hours. One can observe the decrease in growth from T-8 to T-16 in P-35-6; while there was no such trend in the P-35-1C series.

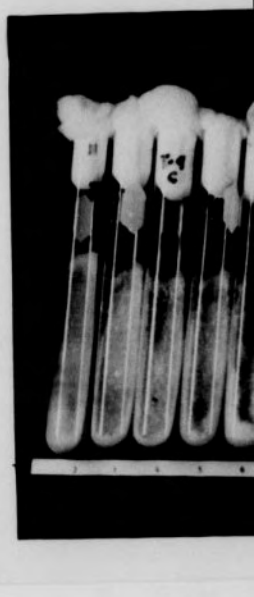
Table 3 gives a mean value and standard error in grams for the wet weight of the mycelia harvested from each flask in each category of strains [SL-3] HC, P-35-6 (T-3 to T-7), P-35-6 (T-8), P-35-6 (T-9 to T-12 plus T-18), P-35-1C (T-3 to T-12 plus T-18). They were divided into categories because it was expected that HC, P-35-6 (T-3 to T-7) and P-35-1C (T-3 to T-12 and T-18) had a wild type cytochrome pattern, while P-35-6 (T-8 to T-12 and T-18) and SL-3 had a [mi-1] type

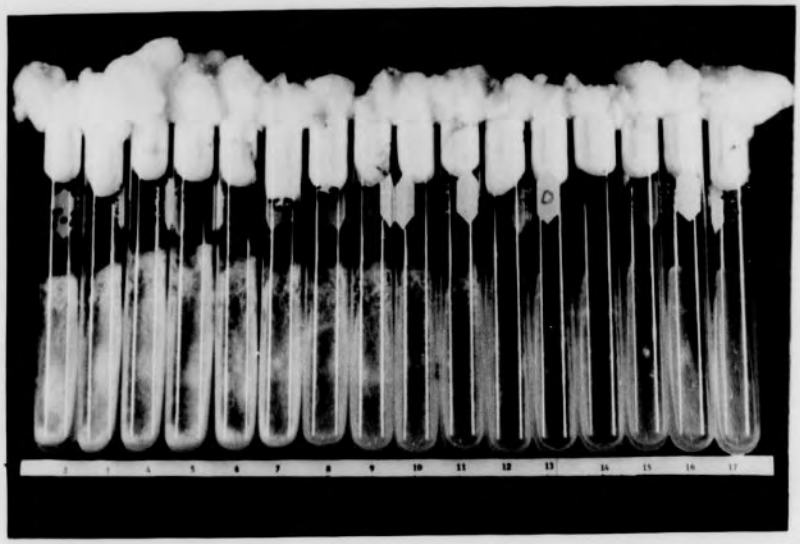
Figure 9

COMPARISON OF GROWTH OF P-35-6 (MICRO-INJECTED)
AND P-35-1C (CONTROL)

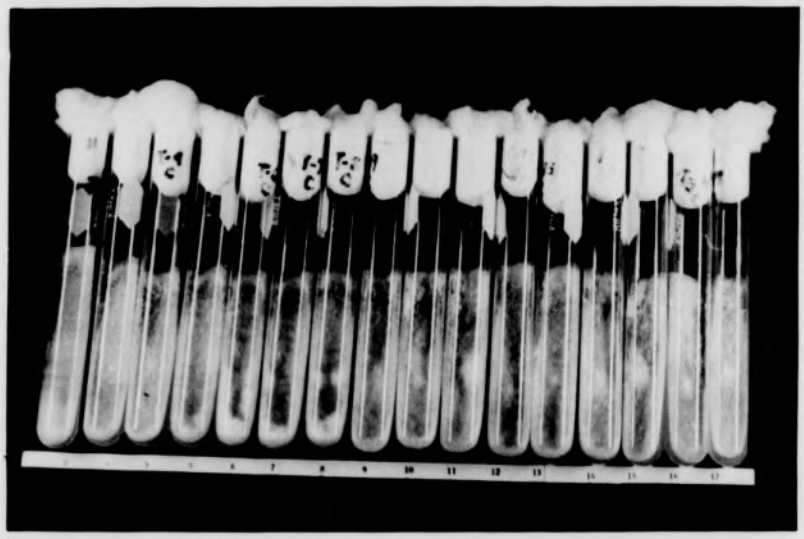
These strains were grown at 30°C for 48 hours, then refrigerated.--(a) P-35-6 transfers 2 to 17.--(b) P-35-1C transfers 2 to 17.







(a)



(b)

TABLE 3

WET WEIGHT AS A FUNCTION OF INOCULATION SIZE AND NUMBER OF FLASKS

	Transfer #	WW in g	# Flasks	g/Flask	Inoc
P-35-6	3	20.50	12	1.72	1X
	4	9.23	10	0.92	1X
	5	13.80	12	1.12	1X
	6	16.13	12	1.34	1X
	7	16.42	18	0.91	1X
	8	18.32	18	1.02	5X
	9	22.40	18	1.24	5X
	10	18.83	18	1.04	5X
	11	15.25	16	0.95	5X
	12	23.50	20	1.17	5X
	18	10.33	19	--	5X
gm/Flask WT	3				
	4	Mean = 1.20 g $\pm$ 0.13 g			
	5				
	6				
	7				
gm/Flask [mi-1]	8	Mean = 1.02 g			
gm/Flask [mi-1]	9				
	10	Mean = 1.10 g $\pm$ 0.06 g			
	11				
	12				
HC (23-8)		18.90	12	1.58	1X
SL-3		6.50	19	0.34	2X
P-35-1C	3	18.26	10	1.83	1X
	4	11.48	10	1.15	1X
	5	10.65	10	1.07	1X
	6	14.64	10	1.46	1X
	7	16.52	12	1.38	1X
	8	19.70	12	1.64	1X
	9	14.90	12	1.24	1X
	10	17.60	12	1.47	1X
	11	18.00	11	1.64	1X
	12	10.74	12	0.90	1X
	18	18.23	12	1.51	1X
		Mean = 1.39 g $\pm$ 0.08 g			

cytochrome pattern. It should be noted that 1 x 10^6 conidia per ml were applied to the liquid media for the strains with a wild type cytochrome pattern and 5 x 10^6 conidia per ml were applied to liquid media for strains with the [mi-1] cytochrome pattern. This was a standard practice to use a 1X inoculum for wild type shaker flasks and a 5X inoculum for [mi-1]. The wet weight was for P-35-6 (T-3 to T-7) 1.20 $\pm$ 0.13 g/flask, for HC 1.58 g/flask, and for P-35-1C 1.39 $\pm$ 0.08 g/flask. These weights showed that P-35-6, HC, and P-35-1C were very similar based on the wet weight per flask with a 1X inoculation. SL-3 had a wet weight of 0.34 g/flask based on a 2X inoculation.

The scans for strains SL-3, HC, P-35-6 (T-3 to T-7), P-35-6 (T-8), P-35-6 (T-9 to T-12 and T-18) and P-35-1C are illustrated in Figures 10, 11, 12, 13, 14, and 15 respectively.

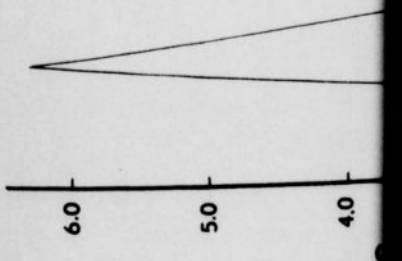
From Figures 10 through 15, it can be seen that HC, P-35-6 (T-3 to T-7), and P-35-1C have the wild type cytochrome distribution; while SL-3, P-35-6 (T-8), and P-35-6 (T-9 to T-12 and T-18), have the [mi-1] cytochrome distribution.

Figure 16 and Figure 17 graphically illustrate α cytochrome c peak height as a function of the amount of protein present for all of the transfers of P-35-6 and P-35-1C scanned.

Figure 18 shows a plot of α cytochrome c peak height as a function of the protein present for P-35-6 (T-3 to T-7), P-35-6 (T-8), and P-35-6 (T-9 to T-12 and T-18). Figure 19 shows the same plot for P-35-1C. From comparison of Figures 18 and 19, it may be

Figure 10

CYTOCHROME ABSORPTION PATTERN SL-3



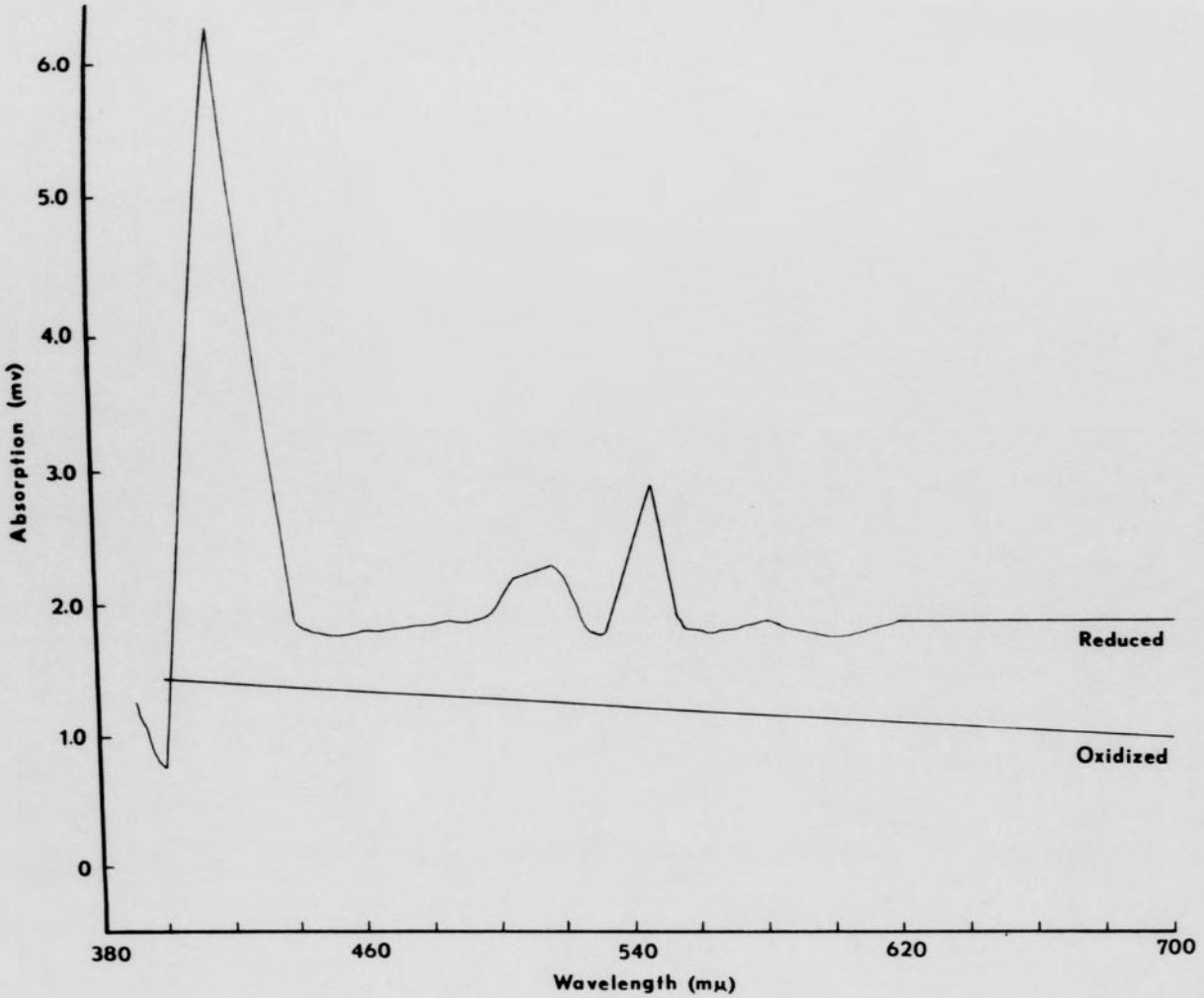
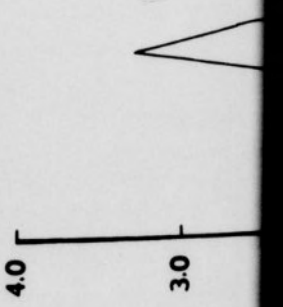

6.0
5.0
4.0
3.0
2.0
1.0
0
Absorption (mv)
Reduced
Oxidized
380
460
540
620
700
Wavelength (mμ)

Figure 11

CYTOCHROME ABSORPTION PATTERN HC



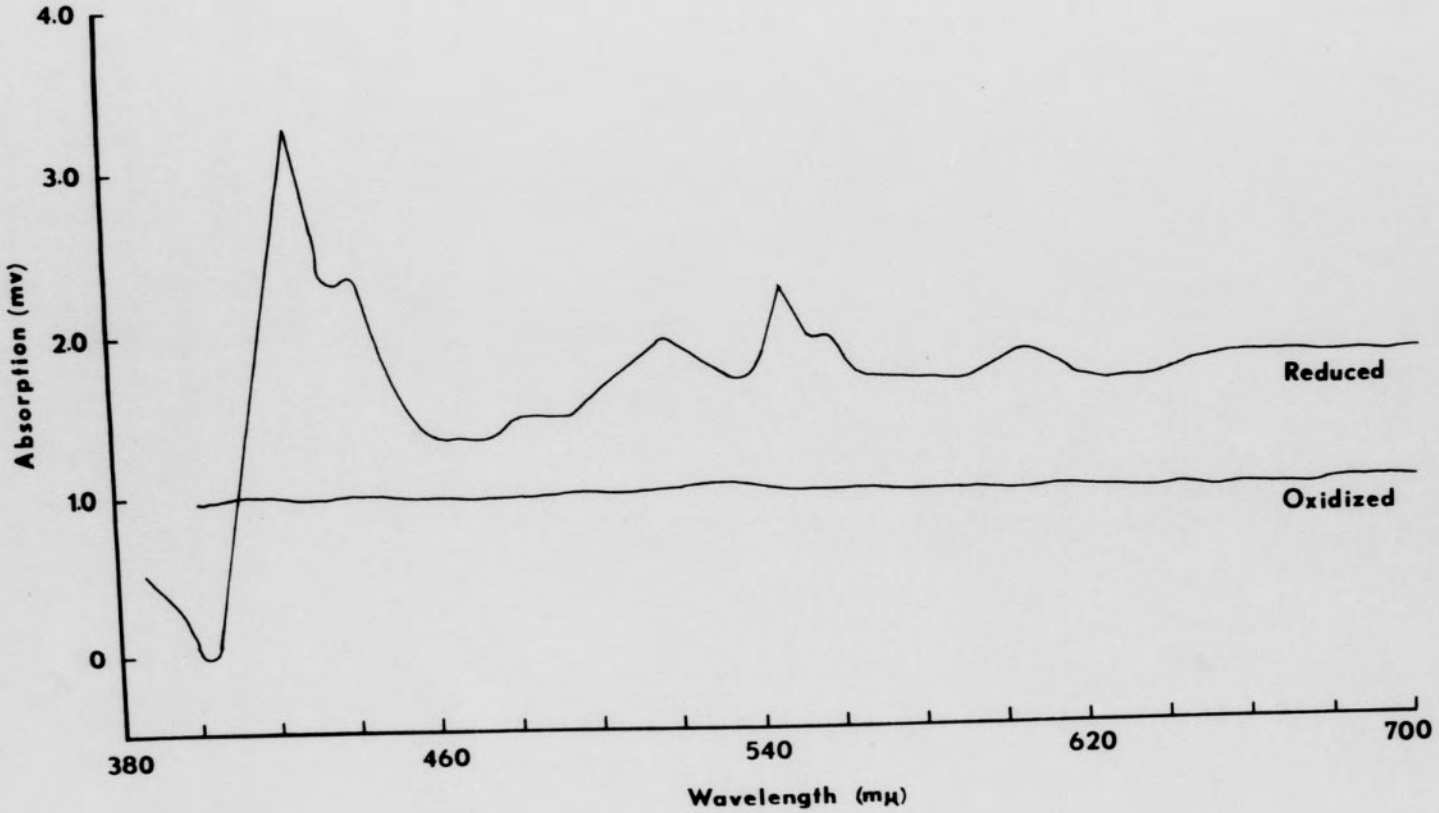
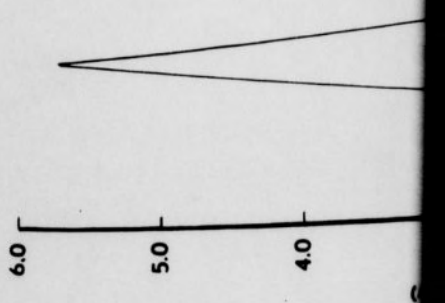

4.0
3.0
2.0
1.0
0
Absorption (mv)
Reduced
Oxidized
380
460
540
620
700
Wavelength (mμ)

Figure 12

CYTOCHROME ABSORPTION PATTERN P-35-6 (T-3 to T-7)



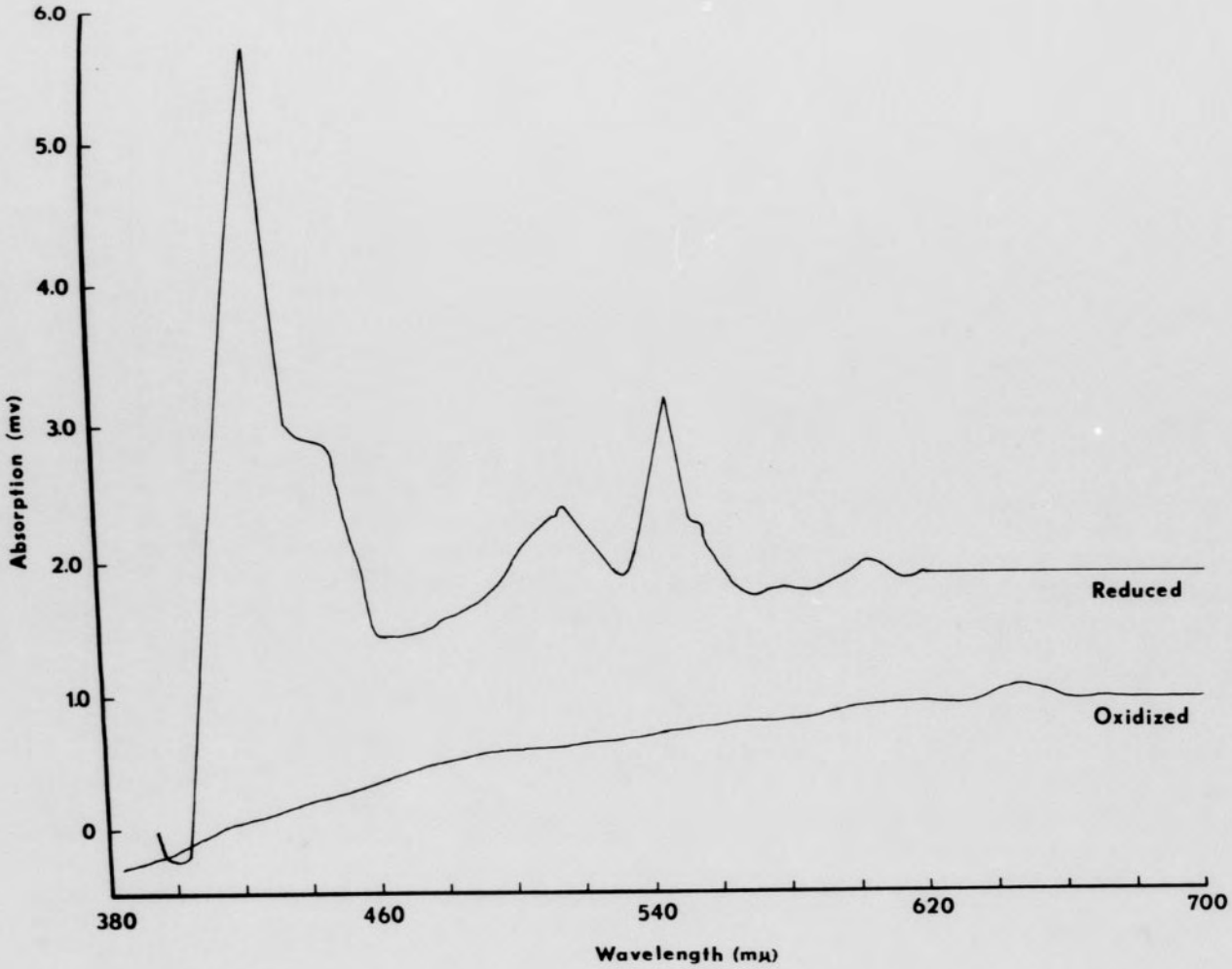
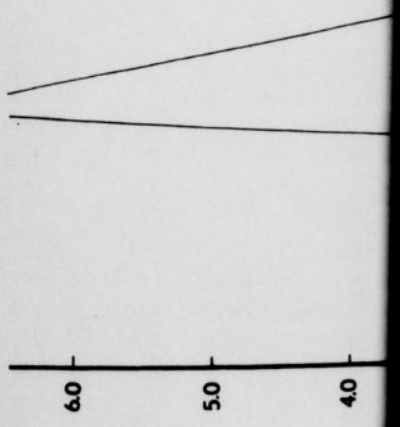

6.0
5.0
4.0
3.0
2.0
1.0
0
Absorption (mv)
Reduced
Oxidized
380
460
540
620
700
Wavelength (mμ)

Figure 13

CYTOCHROME ABSORPTION PATTERN P-35-6 (T-8)



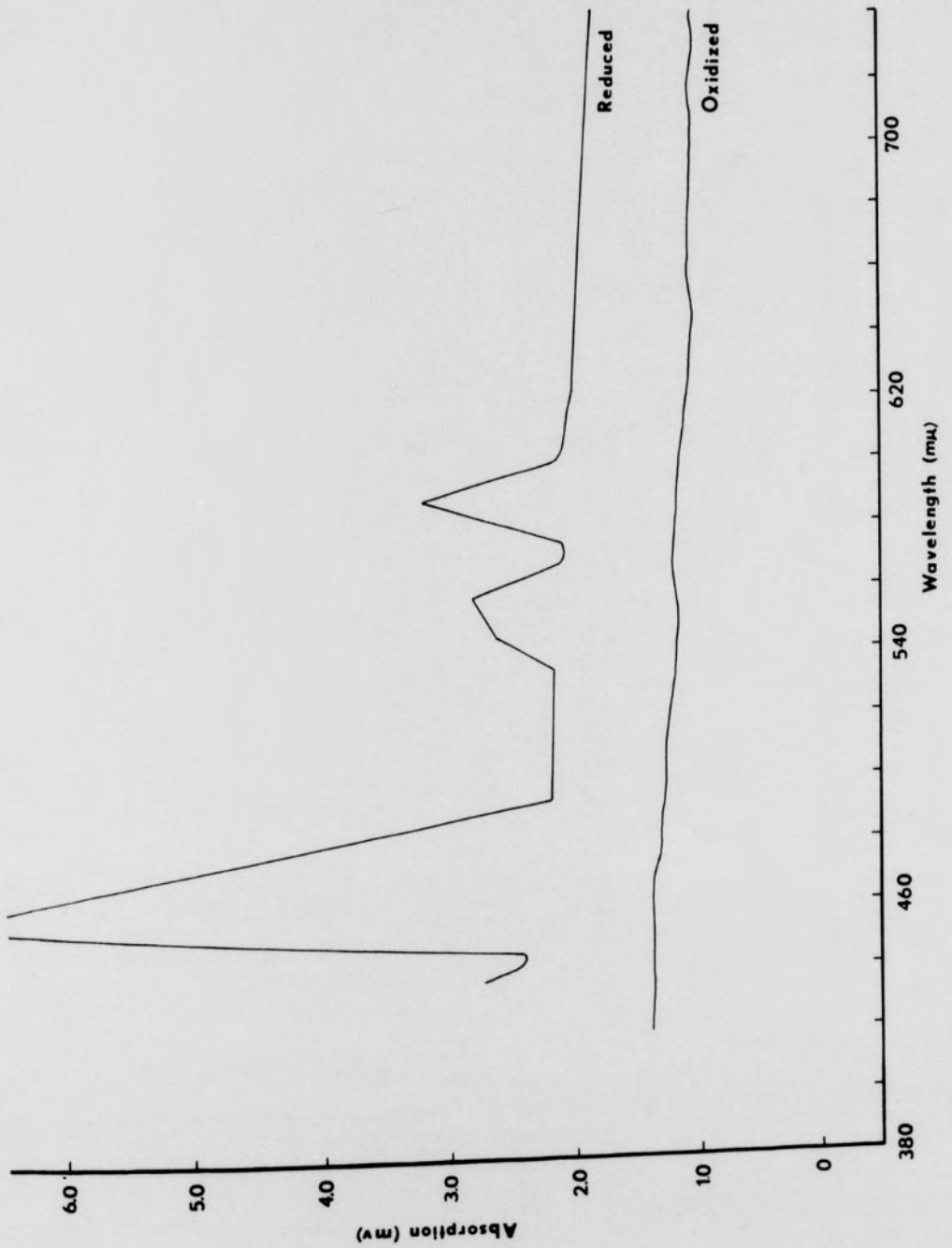
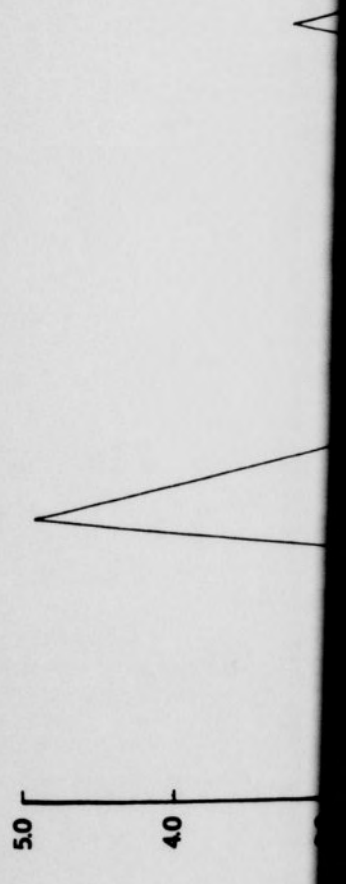

Reduced
Oxidized
Absorption (mv)
6.0
5.0
4.0
3.0
2.0
1.0
0
380
460
540
620
700
Wavelength (mμ)

Figure 14

CYTOCHROME ABSORPTION PATTERN
P-35-6 (T-9 to T-12 AND T-18)



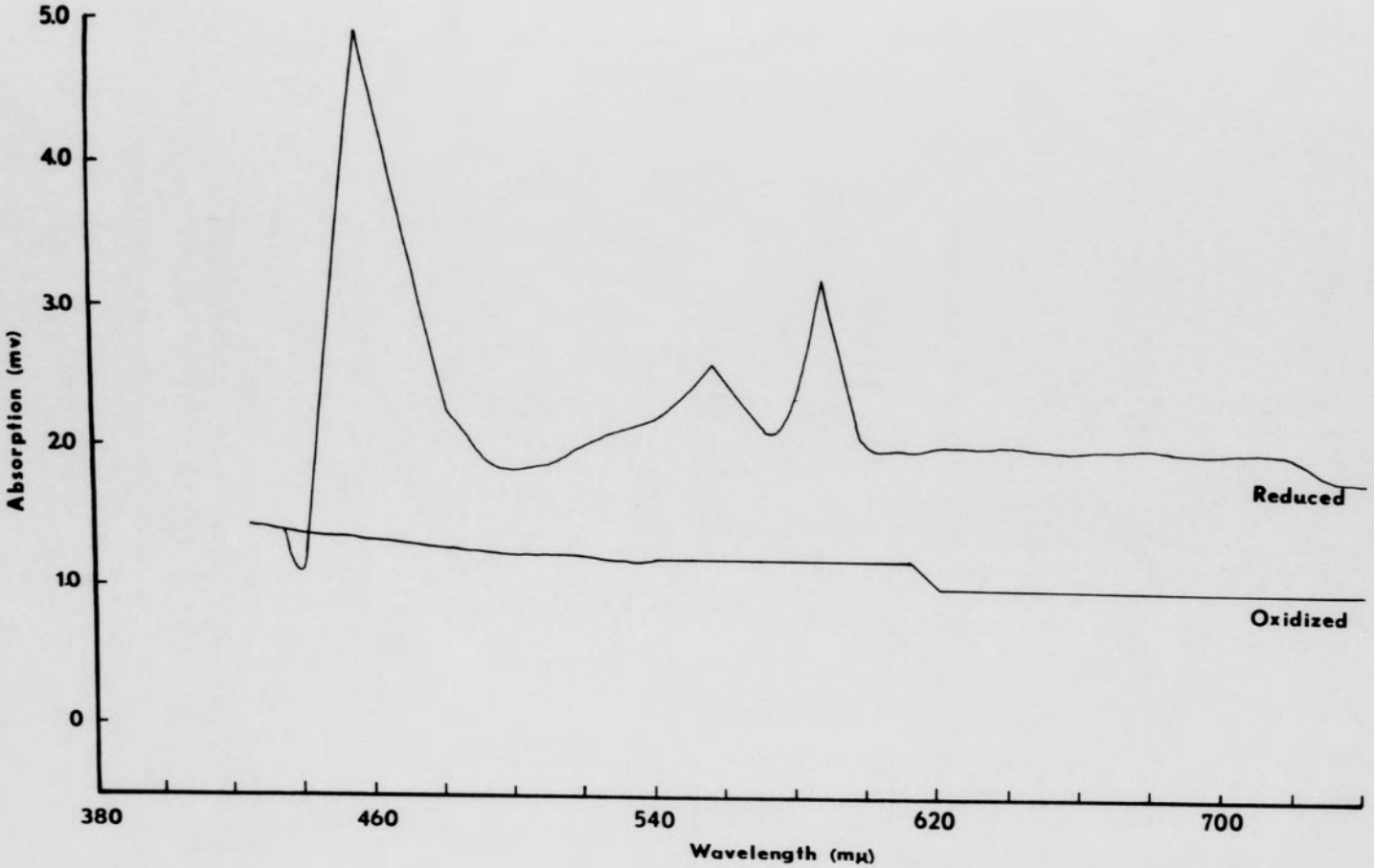
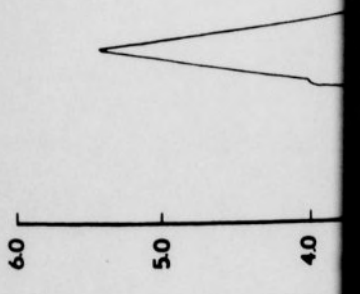

5.0
4.0
3.0
2.0
1.0
0
Absorption (mv)
Reduced
Oxidized
380
460
540
620
700
Wavelength (mμ)

Figure 15

CYTOCHROME ABSORPTION PATTERN P-35-1C



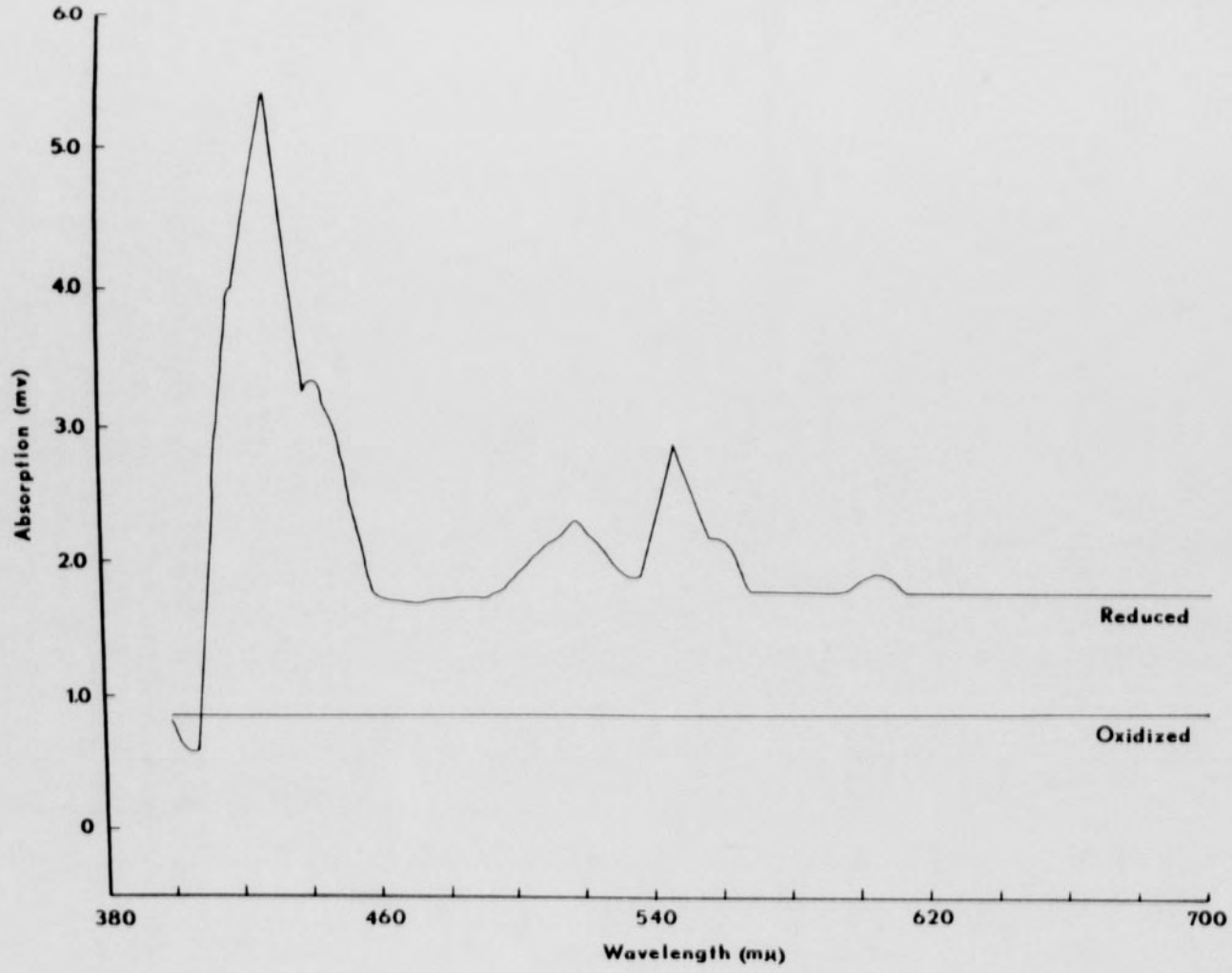

6.0
5.0
4.0
3.0
2.0
1.0
0
Absorption (mv)
Reduced
Oxidized
380
460
540
620
700
Wavelength (mμ)

Figure 16

$\propto$ CYTOCHROME C PEAK HEIGHT AS A FUNCTION OF PROTEIN CONCENTRATION
P-35-6

T number represents serial transfer number.

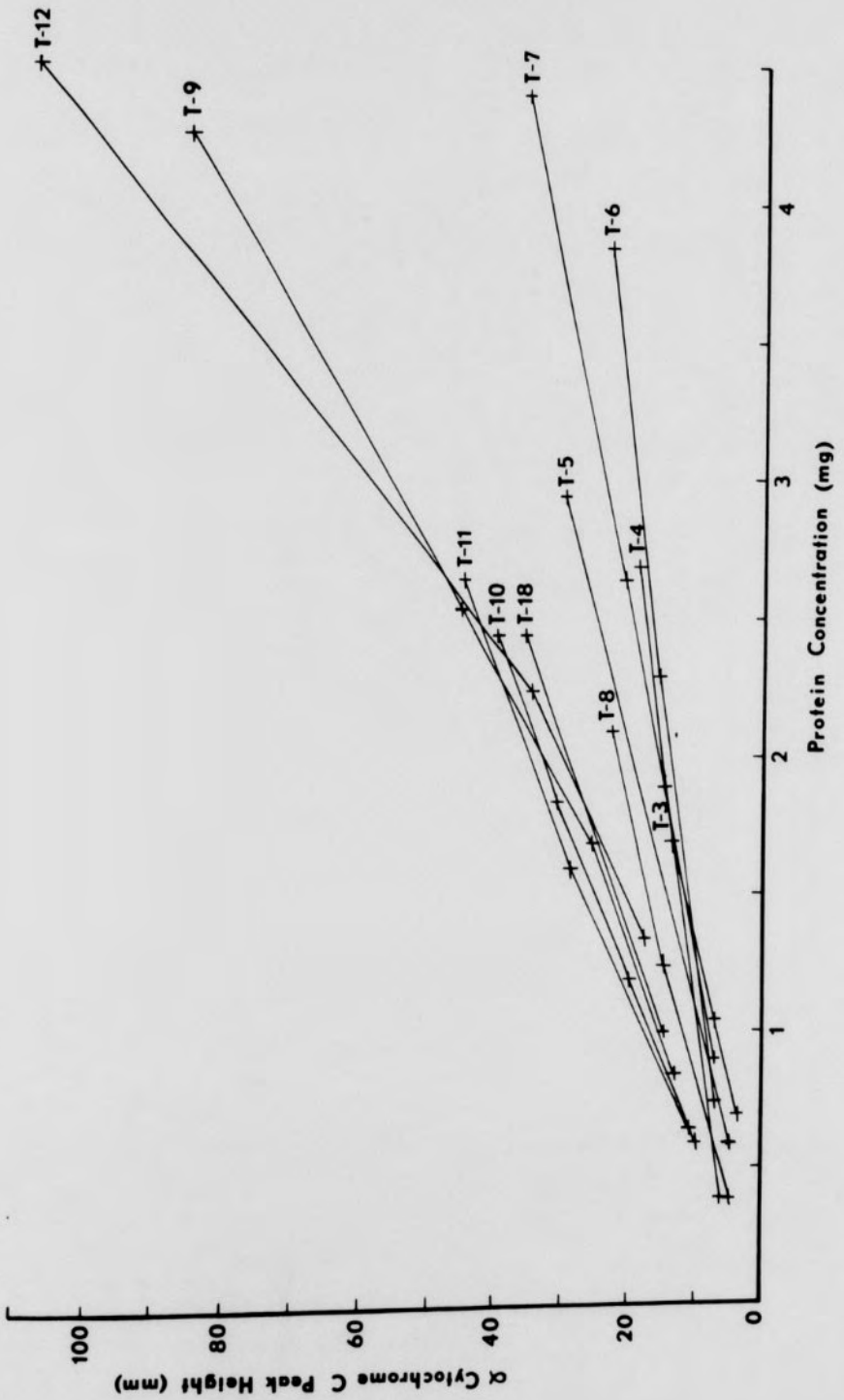

T-12
T-9
T-7
T-6
T-5
T-4
T-11
T-10
T-18
T-8
T-3
100
80
60
40
20
0
1
2
3
4
α Cytochrome C Peak Height (mm)
Protein Concentration (mg)

Figure 17

α CYTOCHROME C PEAK HEIGHT AS A FUNCTION OF PROTEIN CONCENTRATION
P-35-1C

T number represents serial transfer number.

T-8 → T-6

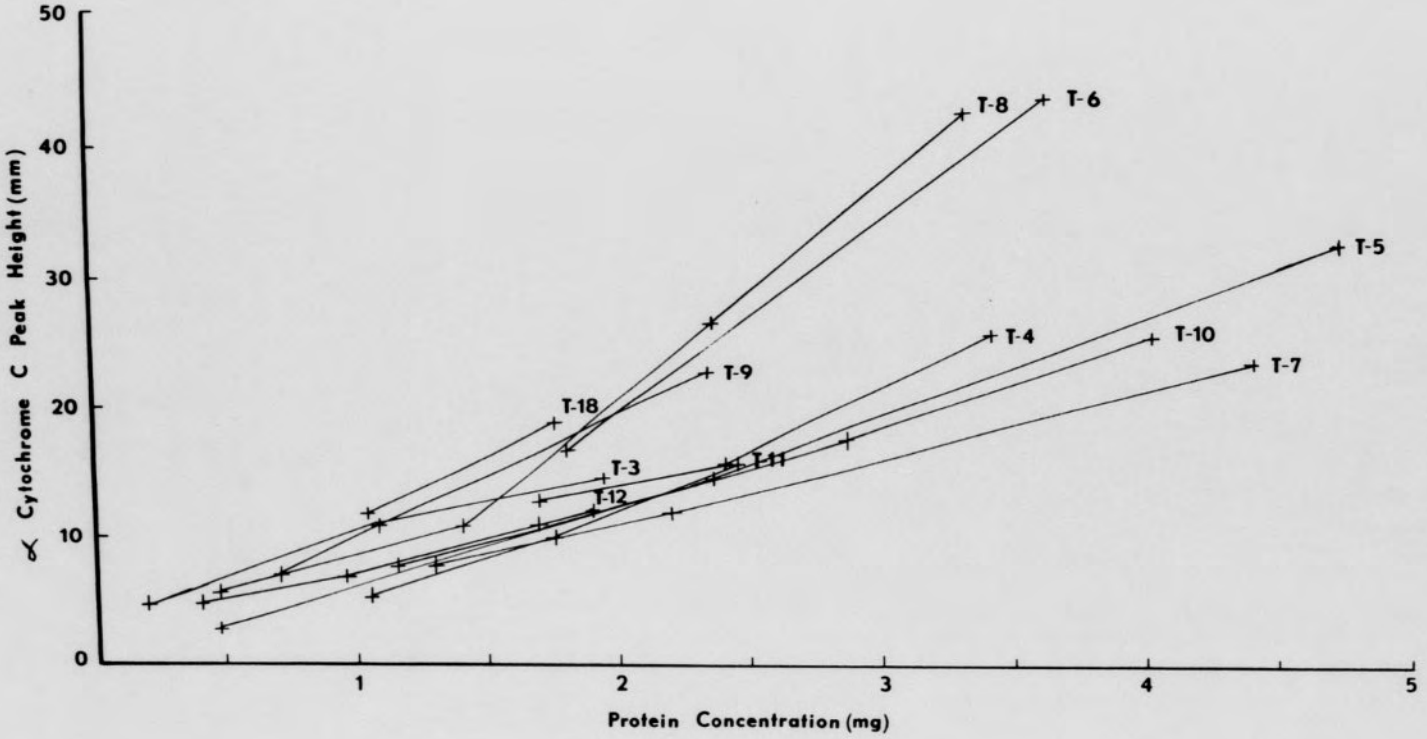

50
40
30
20
10
0
1
2
3
4
5
% Cytochrome C Peak Height (mm)
Protein Concentration (mg)
T-8
T-6
T-5
T-4
T-10
T-7
T-9
T-18
T-3
T-11
T-12

Figure 18

CYTOCHROME C PEAK HEIGHT AS A FUNCTION OF PROTEIN CONCENTRATION
P-35-6 MEAN VALUES

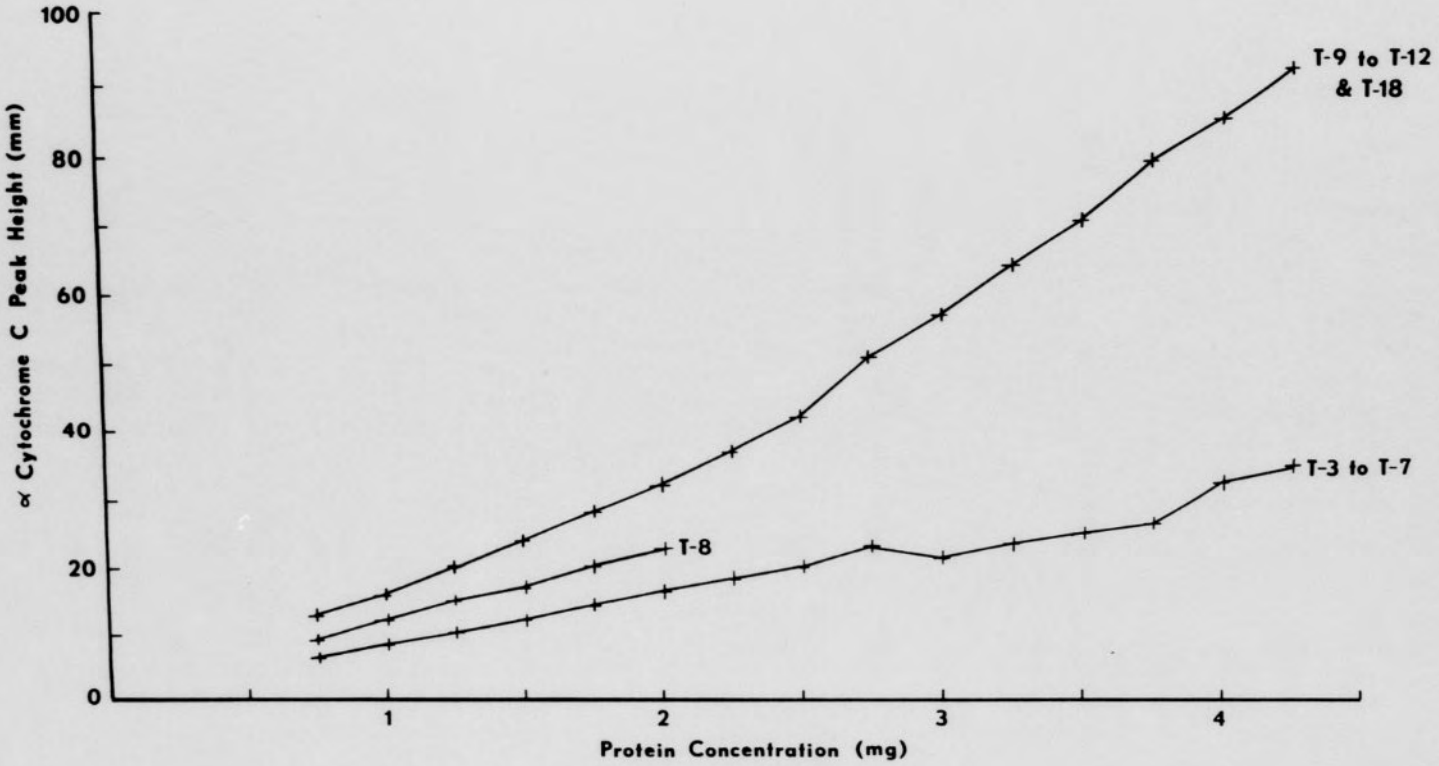
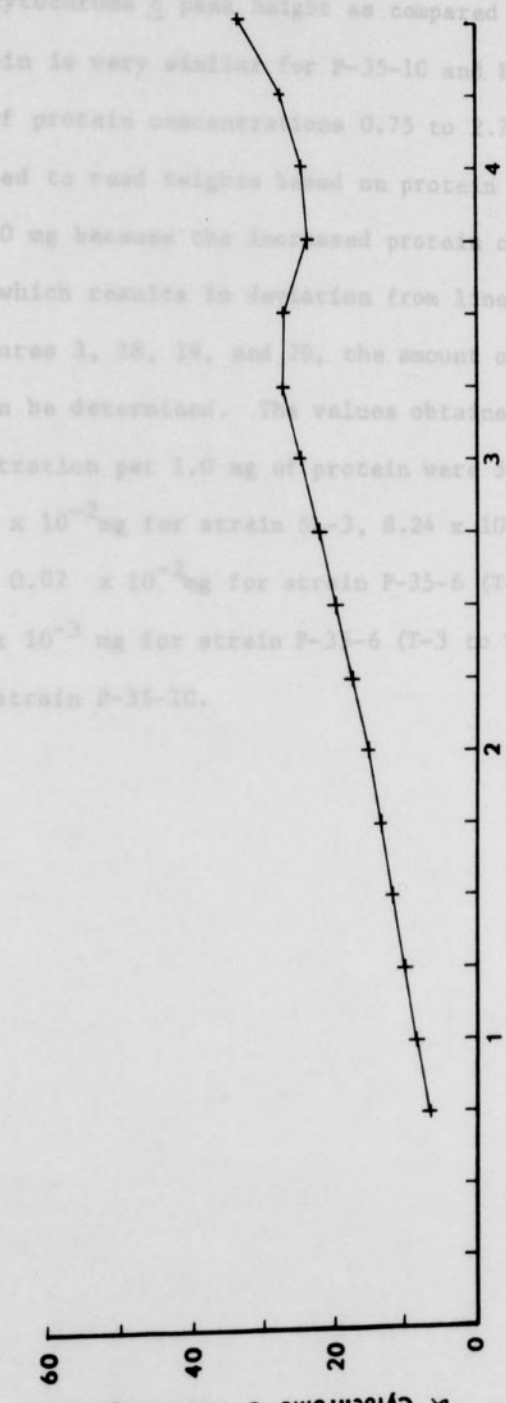

100
80
60
40
20
0
∝ Cytochrome C Peak Height (mm)
1
2
3
4
Protein Concentration (mg)
T-9 to T-12
& T-18
T-3 to T-7
T-8

Figure 19

α CYTOCHROME C PEAK HEIGHT AS A FUNCTION OF PROTEIN CONCENTRATION
P-35-1C MEAN VALUE

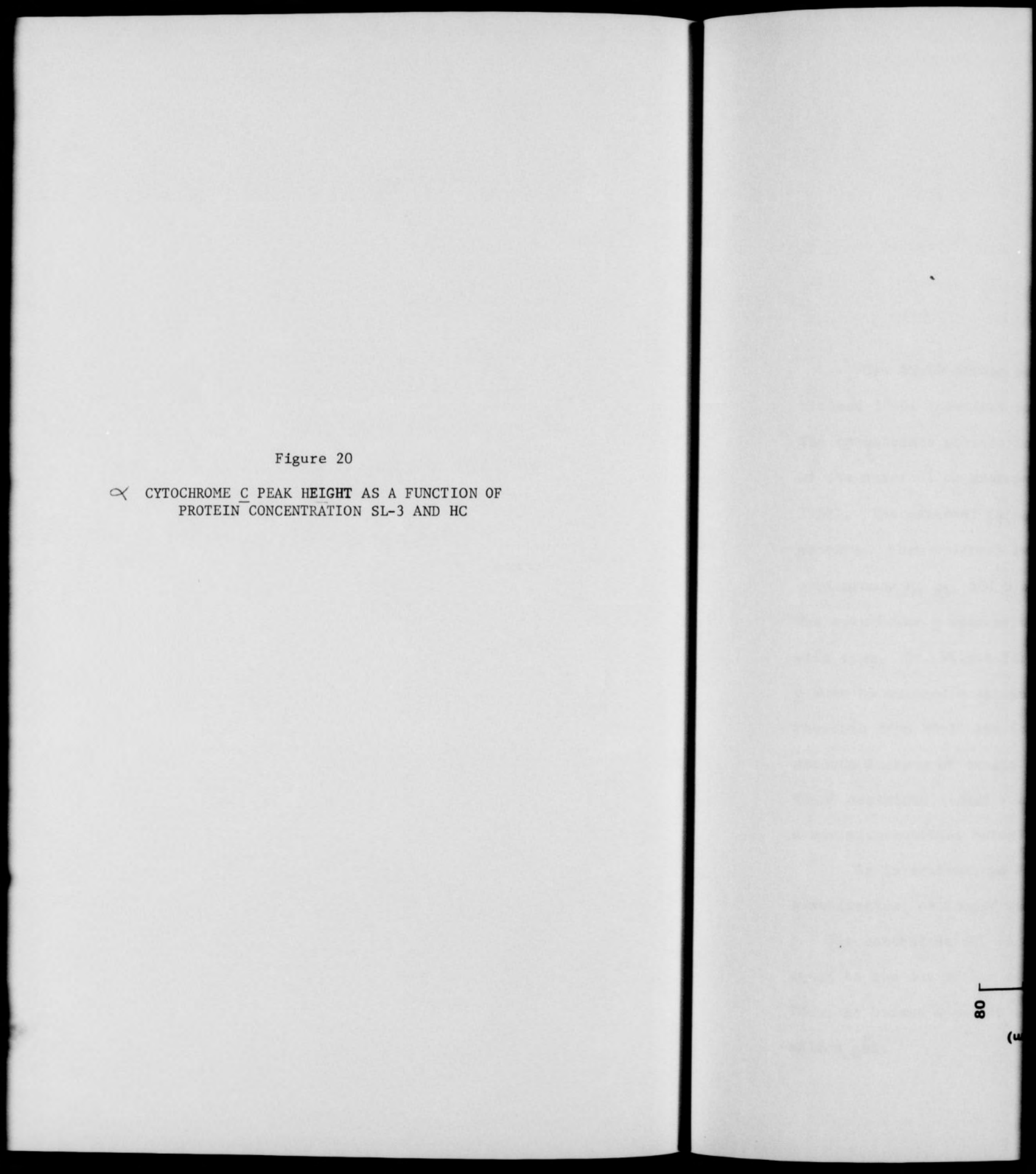

60
40
20
0
1
2
3
4
Protein Concentration (mg)
α Cytochrome C Peak Height (mm)

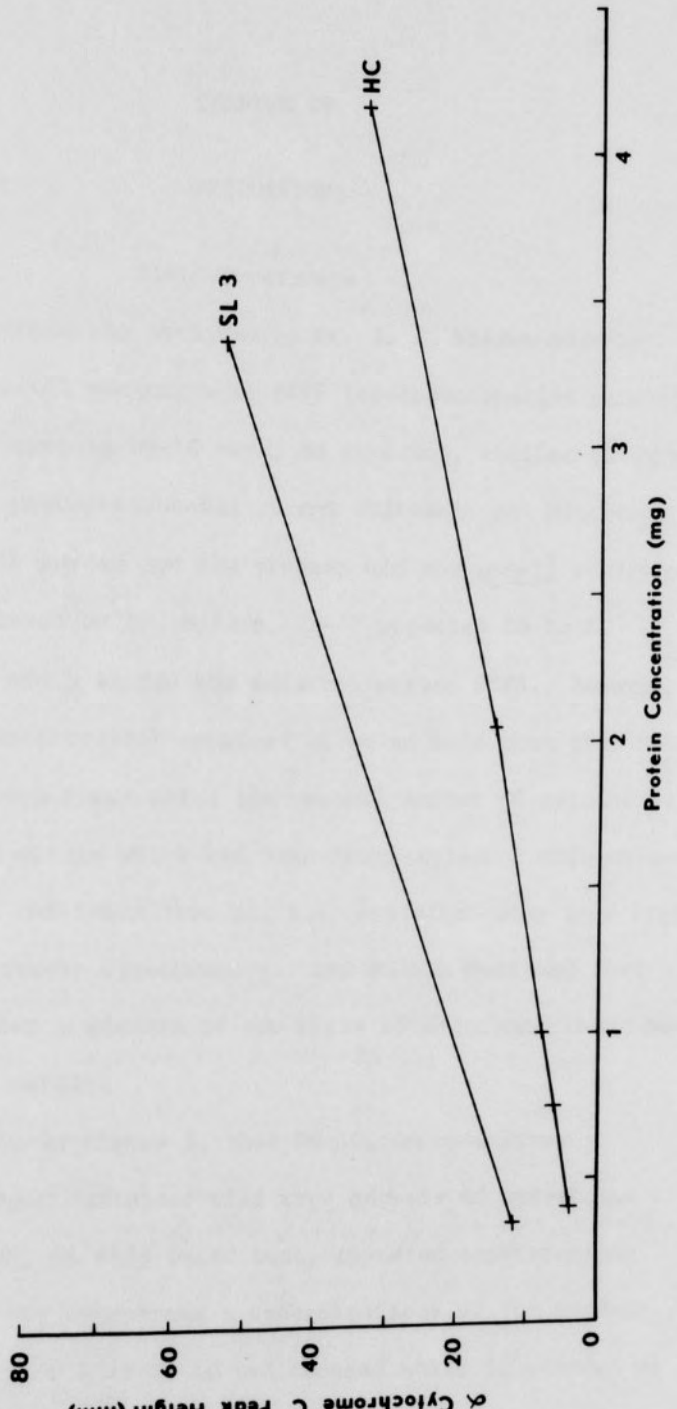
noted that the α cytochrome c peak height as compared to the concentration of protein is very similar for P-35-1C and P-35-6 (T-3 to T-7) in the range of protein concentrations 0.75 to 2.75 mg. Figure 18 should not be used to read heights based on protein concentrations of greater than 2.50 mg because the increased protein concentration causes a turbidity which results in deviation from linearity.

By using Figures 3, 18, 19, and 20, the amount of cytochrome c in each strain can be determined. The values obtained for the cytochrome c concentration per 1.0 mg of protein were 5.90×10^{-3} mg for strain HC, 1.35×10^{-2} mg for strain SL-3, 8.24×10^{-3} for strain P-35-6 (T-8), $1.10 \pm 0.02 \times 10^{-2}$ mg for strain P-35-6 (T-9 to T-12 and T-18), $6.29 \pm 0.20 \times 10^{-3}$ mg for strain P-35-6 (T-3 to T-7) and $6.13 \pm 0.42 \times 10^{-3}$ mg for strain P-35-1C.

Figure 20

CYTOCHROME C PEAK HEIGHT AS A FUNCTION OF PROTEIN CONCENTRATION SL-3 AND HC




SL 3
HC
0
20
40
60
80
1
2
3
4
Protein Concentration (mg)
Cytochrome C Peak Height (mm)

CHAPTER IV

DISCUSSION

NS-10 Experiment

The NS-10 strain was obtained by Dr. J. F. Wilson when he crossed 37401 (conidial parent) with NS#8 (protoperithecial parent). The cytochromes present in NS-10 were, as expected, similar to those of the maternal or protoperithecial parent (Mitchell and Mitchell, 1952). The maternal parent and the progeny had the [mi-1] cytochrome pattern. When observed by Dr. Wilson, NS-10 appeared to lack cytochromes a, a_3, and b as did the maternal parent NS#8. However, the cytochrome c concentration appeared to be no more than that of wild type. Dr. Wilson first noted the unusual amount of cytochrome c when he scanned a strain which had been micro-injected with mitochondria from NS-10 and found that it, too, contained only wild type amounts instead of excess cytochrome c. Dr. Wilson believed that NS-10 contained either a mixture of two types of mitochondria or was a new mitochondrial mutant.

It is evident, in Figure 5, that NS-10, on cytochrome c quantitation, no longer contained wild type amounts of cytochrome c. The concentration, on this later test, appeared approximately equal to the sum of the cytochrome c concentrations of the parents. Thus, it became apparent that NS-10 had changed while in storage on silica gel.

P-35-6 Experiment

The P-35-6 strain was obtained by Dr. J. F. Wilson when he micro-injected [mi-1] mitochondria from SL-3 into HC. SL-3 and HC were of the same mating type, a, but different in their heterocaryon genotype, with SL-3 being Cde, and HC being CDe. Garnjobst and Wilson (1956) stated that heterocaryosis could occur only if the strains were of the same heterocaryon genotype and of like mating type. Wilson et al. (1961) stated that in most strains transfer of protoplasm between incompatible strains resulted in the death of the recipient cell. However, a one gene difference was not as lethal as a two gene difference, and washed mitochondria from an incompatible strain were even less likely to kill. Most of the micro-injected cells survived. Wilson et al. (ibid.) suggested that incompatibility depended on soluble cytoplasmic constituents apparently not associated with subcellular particles. Diacumakos et al. (1965) stated that the incompatibility reaction could provide a biological test for the relative purity of a mitochondrial preparation. In this case, the mitochondria from strain SL-3 (nuclear heterocaryon genotype Cde) did not kill the cell HC (nuclear heterocaryon genotype CDe) on micro-injection.

Wilson (unpublished) noted initially that P-35-6 had a wild type growth rate and a wild type cytochrome distribution. This could be compared to the control series P-35-1C in which he micro-injected mitochondria from wild type strain HC into an HC recipient. Wild type hyphal growth started within a few hours of transfer to the slant. The [mi-1] growth rate lagged about two days behind that of wild type.

On the eleventh serial transfer, P-35-6 displayed the slower [mi-1] growth rate and possessed [mi-1] cytochrome distribution. Diacumakos et al. (1965) stated that there was a considerable and variable time lapse between the injection and appearance of characteristic changes in morphology and growth rate when mitochondria from a maternally inherited mutant [abn-1] were micro-injected into a wild type strain. She suggested that this might be due to the mitochondria or other agents involved having to reproduce and reach a critical level before the changes were observed.

In the present work, the point at which the [mi-1] phenotype was detected in P-35-6 was transfer 8. Thus, this change in phenotype had shifted down from transfer 11 noted by Wilson (unpublished). The growth rate change was also at transfer 8; this can be noted in Figure 10. Wilson had noticed a shift downward of a similar nature in the appearance of [abn-1], which, on silica gel storage, had a shift in the [abn-1] from transfer 8 to the original. Some process in resting conidia caused this downward shift. Three suggestions have been offered to explain how [mi-1] changed the phenotype of the cytochromes in a cell (Diacumakos et al. (1965). These were:

1. A virus introduced with the [mi-1] mitochondria destroyed the wild type mitochondria or converted them to [mi-1].
2. The [mi-1]mitochondria replicated faster and eventually became the predominant species.
3. The [mi-1] mitochondria inhibited reproduction of wild type mitochondria either through the production of some

type of toxic agent or competition for the food source.

Of the preceding possibilities, the least likely is that a virus was introduced with the mitochondria. Freezing and thawing of mitochondrial preparations (which destroy mitochondrial activity but should not destroy viral activity) destroyed the ability of micro-injected [mi-1] mitochondria to change the wild type phenotype of the cell (Wilson, unpublished). It was possible, though improbable, that the virus was either present as an episome or dependent for transfer of whole mitochondria. Also, no viruses have been reported as a result of electronmicroscopic studies of [mi-1] mitochondria.

The data in the RESULTS sections showed that cytochrome c concentration per unit weight of mitochondrial protein was approximately equal for HC, P-35-1C, and P-35-6 (T-3 to T-7). Each of these has the typical wild type cytochrome distribution. The P-35-6 (T-8) had an intermediate value of cytochrome c per mg of protein plus an [mi-1] cytochrome distribution. The P-35-6 (T-9 to T-12 and T-18) had a higher concentration of cytochrome c per mg of protein with a typical [mi-1] cytochrome pattern, but not as high in cytochrome c concentration as SL-3, the strain from which the [mi-1] mitochondria for micro-injection were taken.

Of the remaining two possibilities for [mi-1] action in changing the detectable phenotype, it is difficult to determine which is correct, or whether the [mi-1] dominance is a result of a combination of the two.

Haskins *et al.* (1952) mentioned a cytochrome-destroying enzyme, "cytochromase" produced by [*mi-1*] mitochondria, which destroyed wild type mitochondria *in vitro*. However, this may not be the case *in vivo*.

It would appear that if either of the non-viral explanations were correct, the cytochrome *c* concentration would increase and cytochrome *a*-a_3 and *b* concentrations would decrease gradually through the transfers. However, this was not the case, for there was an abrupt change in cytochrome *c* concentration (T-8) accompanied by a complete disappearance of cytochromes *a* - a_3 and *b* and a reduction in growth rate. If the amount of cytochromes *a* - a_3 and *b* per mg of protein decreased from transfer 1 to transfer 7, this was not detected as the instrumentation did not allow enough sensitivity to quantitate cytochromes *a* - a_3 and *b*. But cytochromes *a* - a_3 and *b* could have been detected if there had not been a great change in their values between transfers 7 and 8.

The pattern of the transition, with the sharp differences between P-35-6 (T-3 to T-7) and P-35-6 (T-9 to T-12 and T-18), suggest some process other than increased growth rate or toxity of the [*mi-1*] mitochondria. These two processes would appear to have caused a gradual shift in phenotype. This unknown process or processes appear to be active even in resting conidia, as shown by the downward shift in the transfer at which the [*mi-1*] phenotype is visible.

With this work it was impossible to determine whether the transition from wild type to [*mi-1*] was influenced by the fact that

[*mi-1*]mitochondria were micro-injected into a heterocaryon-incompatible wild type strain. The complexity of incompatibility would make other studies necessary to clarify the problem.

BIBLIOGRAPHY

Beinert, Helmut. 1951. The extent of artificial redistribution of cytochrome c in rat liver homogenates. J. Biol. Chem. 190: 287-292.

Bensley, R. R. and N. Hoerr. 1934. Studies on cell structure by the freezing-drying method. VI. The preparation and properties of mitochondria. Anat. Record 60:449.

Bertrand, H. and T. H. Pittenger. 1969. An improved method for the determination of mitochondrial cytochromes. N. N. 14:20.

Chance, B. 1951. Spectrophotometric measurements of the cytochrome components of the succinic oxidase system. Fed. Proc. 10:171.

Chance, B. 1952. Spectra and reaction kinetics of respiratory pigments of homogenized and intact cells. Nature 169:215-221.

Chance, B. 1954. Spectrophotometry of intracellular respiratory pigments. Science 120:767-775.

Chance, B. and Benno Hess. 1959. Spectroscopic evidence of metabolic control. Science 129:700-708.

Chance, B. and G. R. Williams. 1956. The respiratory chain and oxidative phosphorylation. Adv. in Enz. 17:65-134.

Claude, Albert. 1946. Fractionation of mammalian liver cells by differential centrifugation. J. Exptl. Med. 84:51-61.

Diacumakos, E. G., L. Garnjobst and E. L. Tatum. 1965. A cytoplasmic Character in Neurospora crassa. J. Cell Biol. 26:427-443.

Dickerson, Richard E. and Irving Geis. 1969. The structure and action of protein. Harper and Row, New York, 120 p.

Ephrussi, B. and H. Hottinguer. 1951. Cytoplasmic constituents of heredity on an unstable cell state in yeast. Cold Spring Harbor Symposia Quant. Biol. 16:75-85.

Garnsjobst, L. and J. F. Wilson. 1956. Heterocaryosis and protoplasmic incompatibility in Neurospora crassa. Proc. Nat. Acad. Sci. 42 (9):611-618.

Garnjobst, L., J. F. Wilson and E. L. Tatum. 1965. Studies on a cytoplasmic character in Neurospora crassa. J. Cell Biol. 26:413-425.

Gornall, Allan G., Charles J. Bardawill and Maxima M. David. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177:751-766.

Griffiths, A. J. F., H. Bertrand and T. H. Pittenger. 1968. Cytochrome spectra of cytoplasmic mutants in Neurospora. N. N. 13:16-17.

Haskins, Francis A., Alfred Tissieres, Herschel K. Mitchell and May B. Mitchell. 1953. Cytochromes and the succinic acid oxidase system of poky strains of Neurospora. J. Biol. Chem. 200-819-826.

Heller, Joram and Emil L. Smith. 1966. Neurospora crassa cytochrome c. II. Chymotryptic peptides, tryptic peptides, cyanogen bromide peptides and the complete amino acid sequence. J. Biol. Chem. 241:3165-3180

Hogeboom, George H., Walter C. Schneider and George E. Pallade. 1948. Cytochemical studies of mamalian tissues. I. Isolation of intact mitochondria from rat liver; some biochemical properties of mitochondria and submicroscopic particulate material. J. Biol. Chem. 172:619-636.

Keilin, D. 1925. On cytochrome, a respiratory pigment, common to animals, yeast, and higher plants. Roy. Soc. (London), Proc., B. 98:312-339.

Keilin, D. 1930. Cytochrome and intracellular oxidase. Roy. Soc. (London), Proc., B. 106:418-444.

Keilin, D. and E. F. Hartree. 1939. Cytochrome and cytochrome oxidase. Roy. Soc. (London), Proc., B. 127:167-191.

Kennedy, Eugene P. and Albert L. Lehninger. 1948. Intracellular structures and the fatty acid oxidase system of rat liver. J. Biol. Chem. 173:847-848.

Luck, David J. L. 1965. The influence of precursor pool size on mitochondrial composition in Neurospora crassa. J. Cell Biol. 24:445-460.

MacMunn, C. A. 1886. Researches on myohaematin and the histo-haematins. Phil. Trans. 177:267-298.

Millikan, G. A. 1937. Experiments on muscle haemoglobin in vivo; the instantaneous measurement of muscle metabolism. Roy Soc. (London), Proc., B. 123:218-241.

Mitchell, M. B. and Herschel K. Mitchell. 1952. A case of "maternal" inheritance in Neurospora crassa. Proc. Nat. Acad. Sci. 38: 442-449.

Mitchell, M. B., H. K. Mitchell and Alfred Tissieres. 1953. Mendelian and non-mendelian factors affecting the cytochrome system in Neurospora crassa. Proc. Nat. Acad. Sci. 39:606-6B .

Potter, V. R. 1941. Studies on the mechanism of hydrogen transport in animal tissues. III. Cyanide inhibition of cytochrome c reduction. J. Biol. Chem. 137:13-20.

Potter, V. R. and K. P. DuBois. 1942. The quantitative determination of cytochrome c. J. Biol. Chem. 142:417-426.

Schneider, Walter C. 1946. Intracellular distribution of enzymes. I. The distribution of succinic dehydrogenase cytochrome oxidase, adenosinetriphosphatase, and phosphorus compounds in normal rat tissues. J. Biol. Chem. 165:585-593.

Schneider, Walter C. and G. H. Hogeboom. 1950. Intracellular distribution of enzymes. V. Further studies on the distribution of cytochrome c in rat liver homogenates. J. Biol. Chem. 183:123-128.

Stotz, Elmer. 1939. The estimation and distribution of cytochrome oxidase and cytochrome c in rat tissues. J. Biol. Chem. 131:555-565.

Tissieres, Alfred and Herschel K. Mitchell. 1954. Cytochromes and respiratory activities in some slow growing strains of Neurospora. J. Biol. Chem. 208:241-249.

Vogel, H. J. 1956. A convenient growth medium for Neurospora. Microbiol. Genet. Bull. 13:42-43.

Wagner, Robert P. and Herschel K. Mitchell. 1964. Genetics and metabolism. John Wiley and Sons, Inc., New York. 673 p.

Wilson, J. F. 1961. Microsurgical techniques for Neurospora. Amer. J. Bot. 48:46-51.

Wilson, J. F., L. Garnjobst and E. L. Tatum. 1961. Heterokaryon incompatibility in Neurospora crassa. Micro-injection studies. Amer. J. Bot. 48:299.

Woodward, D. O. and K. D. Munkres. 1966. Alterations of a maternally inherited mitochondrial structural protein in respiratory-deficient strains of Neurospora. Proc. Nat. Acad. Sci. 55:872-880.