CYTOCHROME c SYNTHESIS IN NEUROSPORA crassa

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Abstract

A. Cytochrome c synthesis was studied in the respiration deficient poky mutant of Neurospora crassa. Two cytochromes c, C_I and C_{II}, were detected. The two proteins were shown to differ by a secondary modification at residue 72. A precursor-product relationship was established; C_{II} (lysine) is converted to C_I (Lys-X). Lys-X was found to be a lysine derivative although the exact chemical nature of this residue is unknown.

The kinetics of C_I and C_{II} synthesis in poky were compared to those of wild type and the respiration deficient mutants: mi-3, cyt-1, and po-f. In comparison to wild type, poky accumulates C_{II} during the early stages of growth and has a delayed synthesis of C_I. These events in the other mutants are normal even though they accumulate cytochrome c as does poky.

Only C_I was recovered from isolated poky mitochondria even though the whole cell contained both cytochromes c. In young poky over half of the cytochrome c is unbound. The conversion of C_{II} to C_I is thought to reflect a binding of cytochrome c to mitochondria since this event parallels the change of the poky phenotype to a more normal state.

B. When wild type Neurospora is grown in high concentrations of chloramphenicol (4 mg./ml.), it exhibits a phenotype similar to that of the maternally inherited poky mutation. Both have no cytochromes a and b but have an excess of cytochrome c. Spontaneously occurring strains

iv

partially resistant to the drug were isolated. The resistance is controlled by more than one nuclear gene. The implications of these results are discussed in terms of the primary effect of the poky mutation and the differential inhibition of mitochondrial protein synthesis by chloramphenicol in cells of higher organisms.

v

TABLE OF CONTENTS

1

INTRODUCTION

Although cytochrome c has been studied intensively from a physico-
chemical approach (1), little attention has been given to its biosynthe-
sis. The primary structure of cytochrome c is encoded by a nuclear gene
(2), and it is thought to be synthesized on cytoplasmic ribosomes (3,4,5).
The details of cytochrome c m-RNA translation on cytoplasmic ribosomes to
form the polypeptide chain, although unknown, are undoubtedly the same
as for other proteins. Cytochrome c, however, is especially interesting
because it occurs in vivo as an integral part of the electron transport
chain in mitochondria (1). As a result cytochrome c synthesis is inti-
mately related to mitochondrial biogenesis. According to Luck (6), Neuro-
spora mitochondria grow by accretion of precursors from the cytoplasm.
Cytochrome c, therefore, must be packaged into the mitochondrial struc-
ture by some unknown mechanism. Assuming a constant amount of cytochrome
c per mitochondrial mass, mitochondrial biogenesis and cytochrome c synthe-
sis must be coordinated since, under normal circumstances, the protein is
not found free in the cellular cytoplasm. In this context the cytoplasmi-
cally inherited poky character of Neurospora is important. The poky mutant
accumulates cytochrome c (compared to wild type) (12) and, in addition,
over half of the cytochrome c is unbound (7). This implies that the
mechanism to integrate cytochrome c and/or the control of cytochrome c
synthesis in poky is in disarray.

The biosynthesis of cytochrome c was investigated in the poky mu-
tant of Neurospora, mainly for the above reasons. Since many aspects of
cytochrome c synthesis and mitochondrial biogenesis are still controver-
sial each is discussed briefly. In addition, the respiration deficient mu-

tants of Neurospora are described and a few comments are included on cytochrome c structure that are relevant to the results presented here.

Respiration Deficient Mutants of Neurospora

Mutations in yeast (8) and Neurospora (9) that affect mitochondrial function are well known. Two of the best studied mutations of Neurospora, mi-1 (poky) and mi-3, were isolated by Mitchell and Mitchell (10,11) and were shown to be maternally inherited. Crosses between poky or mi-3 and wild type using the mutants in all combinations as perithecial parents yield progeny only of the mutant phenotype. The reciprocal cross yields only wild type progeny.

Both mi-3 and poky are characterized by slow growth and abnormalities of their respiratory systems. Young poky accumulates up to sixteen times the amount of cytochrome c found in wild type and lacks cytochromes b and a (12). Mi-3 also accumulates cytochrome c (only 2.4 times that of wild type), lacks cytochrome b, and in addition contains cytochrome a_1 unlike either poky or wild type (13). The poky phenotype changes with age; young poky contains no cytochromes a and b, but as the culture ages the phenotype becomes more like wild type (12). In old poky cultures the amount of cytochrome c per unit mass of tissue decreases, and cytochromes a and b are found. It should be noted, however, that poky never becomes entirely normal and these changes in cytochrome content are repeated through successive transfers. A nuclear modifier gene has also been described for poky (14). The effect of this gene is to increase the growth rate of poky, reduce the cytochrome c accumulation but not to affect the lack of cytochromes a and b. The f gene, however, has no effect on any of the other respiratory mutants.

Tatum and co-workers (15) have described two Neurospora mutants, abn-1 and abn-2, which are characterized by irregular growth and excessive amounts of cytochrome c plus a lack of cytochrome b. These strains are female sterile since they produce no protoperithecia. However, crosses of both abn-1 and abn-2 as the male parent with wild type produce only normal progeny. The mutants, nevertheless, have been transmitted through heterocaryons to other strains. These facts are reminiscent of the poky and mi-3 mutants and suggest that these two characters are also transmitted by cytoplasmic factors. Furthermore, microinfected mitochondria isolated in a sucrose gradient from abn-1 conveyed the mutant characteristics to wild type Neurospora after several transfers of the injected strain (16). Injected nuclei of abn-1 had no effect. Thus, mitochondria can and do transmit cytoplasmically inherited traits.

In addition to these maternally inherited mutations, two nuclear genes are known to affect the respiratory system of Neurospora. Cyt-1 (C-115) (11) accumulates cytochrome c, has a normal complement of cytochrome a and lacks cytochrome b (13). Cyt-2 (C-117) (11) differs from cyt-1 since it lacks both cytochromes a and c but contains cytochrome b and cytochrome e, which is not found in wild type or any of the other mutants (13).

Another characteristic of the respiratory mutants was described by Woodward and Munkres (17). Comparative studies were carried out on the mitochondrial structural protein (MSP) isolated from wild type, poky and mi-3. Compared with wild type MSP, the poky protein was found to have a single amino acid replacement of cysteine for tryptophan. MSP from mi-3 also lacks one of the three tryptophan residues but does not have an extra

cysteine. However, cyt-1 and cyt-2 (both nuclear mutants) have apparently normal MSP as ascertained by amino acid analyses. Interesting also was the finding that pof MSP has the same Tryp→Cys change as poky. The f gene must compensate for the defective MSP by, perhaps, an increase in activity of some other enzyme system as proposed by Mitchell and Mitchell (14). The pleiotropic effect of the poky mutation can be explained on the assumption that MSP plays a critical role in the organization and assembly of the electron transport system (19). This assumption is based on the observations of Criddle et al. (20,21) that MSP (beef heart) will bind cytochromes c, c_1, b and a in a 1:1 stoichiometric complex and also interacts with phospholipids (22). The amino acid replacement in the poky and mi-3 MSP was found to cause the protein to differ from that of wild type in binding properties with coenzyme nucleotides and malate dehydrogenase. Hence, genetic alterations of MSP leading to decreased affinity for proteins that are directly or indirectly associated with the electron transport system may disrupt the assembly of this complex (17).

Some reservation about the work with structural protein should be voiced. Because of the marked insolubility of the protein, characterization has been difficult. Nevertheless, Criddle et al. (23) have found a molecular weight of 22,000 for beef heart MSP calculated from amino acid analyses (20). This is in good agreement with the number of tryptic peptides and the number of N-terminal amino acids (one N-acetyl serine per 22,500 molecular weight). Similar agreement between the number of tryptic peptides and amino acid composition was reported for Neurospora MSP (18). Because of the similarity between the solubility of MSP and

his F-4 coupling particle, Racker compared the ability of the two to restore phosphorylation in submitochondrial particles. Both restore P^{32}-ATP exchange and oxidative phosphorylation, but neither had an effect on oxygen uptake (24). In addition, both MSP and F-4 bind phospholipids and cytochrome b equally well at neutral pH. Haldar et al. (25), however, have compared MSP isolated from rat liver and Racker's coupling factor 4 by acrylamide gel electrophoresis. They found the MSP preparation gave three major bands, while the F-4 coupling factor gave one. The difference between the two preparations is in the method of isolation; Racker's F-4 coupling factor is isolated by sonicating mitochondria in the presence of phosphotides (26), while MSP is isolated by extracting mitochondria with anionic detergents (22). Assuming the two procedures are extracting the same protein, the studies with MSP should be viewed with some reservation until the question of MSP homogeneity and "nativeness" is settled.

Woodward and Munkres (19) have detected structural protein in isolated mitochondria, nuclei, microsomes and 100,000 x g supernatant fraction of Neurospora. Structural protein isolated from all subcellular fractions of poky and mi-3 has the same amino acid substitution as did the MSP. Although these structural proteins have similar amino acid compositions, tryptic peptide maps show specific differences, especially between the nuclear and mitochondrial proteins. To explain how the same amino acid substitution can occur in structural protein throughout the cell, Woodward and Munkres have hypothesized (19) that structural protein is synthesized in mitochondria then released into the cellular cytoplasm. Although this is an attractive hypothesis, it cannot be accepted until it

has been proven that the structural proteins isolated from the different cellular fractions are identical.

Recent reports have indicated that drugs which are effective inhibitors of bacterial protein synthesis cause normal aerobically growing yeast to exhibit a phenotype similar to that of the respiration deficient petite mutant (27,28). Most of these experiments were carried out with chloramphenicol. Chloramphenicol is a well known inhibitor of protein synthesis although the site of its action is unclear. Present evidence indicates that it acts at a stage after binding of m-RNA to the ribosome and during peptide bond synthesis (29). It is known that concentrations of 10^{-4} to 10^{-5} $\underline{M}$ chloramphenicol which effectively inhibit bacterial protein synthesis have no effect on cell free systems of animals (30) and yeast (31,32) nor on the growth of Neurospora (33). On the other hand, chloramphenicol has been shown to inhibit amino acid incorporation by isolated mitochondria from yeast (34) and from mammalian sources (35). These observations together with the phenotypic effect of the drug have led to the hypothesis that chloramphenicol treatment affects the same system in yeast as does the cytoplasmic mutation of the petite mutant (92).

Biogenesis of Mitochondria

Evidence for the genetic continuity of the mitochondrion first came from the respiration deficient poky and mi-3 mutants of Neurospora (9) and the petite mutants of yeast (8). Genetic and biochemical experiments leave no doubt that the difference in phenotype between each of these mutants and wild type is localized in the mitochondrion and is extrachromosomal in origin. Recently Luck (6) has provided direct evidence for the

genetic continuity of mitochondria by demonstrating that preexisting

Neurospora mitochondria may grow and divide. Luck labeled cells of a

choline-requiring strain with radioactive choline then transferred them

to unlabeled medium. At various times during the subsequent logarithmic

growth, mitochondria were isolated. The distribution of label among in-

dividual mitochondria was determined by autoradiography. Analyses demon-

strated that the choline label was randomly distributed among mitochondria

of fully labeled cells as well as cells undergoing logarithmic growth.

At the end of each doubling cycle and through at least three consecu-

tive doubling cycles, the average grain count per mitochondrion had

decreased by one-half that found at the end of the preceding cycle.

From these observations Luck concluded that mitochondria grow by

random accretion of choline and other precursors to existing mitochondria,

then multiply by division. Additional support for Luck's conclusions

comes from recent findings that mitochondria contain a constant amount of

DNA (36,37,38,99), and the necessary apparatus for DNA replication (39).

Mitochondrial DNA, however, does not contain enough information to code

for the primary structures of all proteins known to be associated with the

mitochondrion. DNA of a molecular weight of 13×10^6 daltons (Neurospora

mitochondrial DNA (37)) can code for about 20 proteins with a molecular

weight of 30,000. C. de Duve et al. (40) list about 50 enzymes that are

known to be associated with or partially associated with mitochondria.

Therefore, over half the mitochondrial enzymes are encoded by nuclear

genes. This conclusion is also supported by the observation that muta-

tions affecting several mitochondrial enzymes such as malate dehydrogenase,

aspartate amino transferase (41) and cytochrome c (2) segregate in a nor-

mal Mendelian fashion.

Site of Cytochrome c Synthesis

Current thought is that cytochrome c is synthesized in the cyto-
plasm then transferred to mitochondria. As yet no conclusive experiments
have demonstrated a de novo synthesis on cytoplasmic ribosomes. When
mitochondria are incubated with labeled amino acids in vivo, the label is
incorporated into an insoluble lipoprotein-rich fraction only (42).
Extensive work by Roodyn, Work and co-workers (5) has shown that solu-
ble enzymes such as catalase, malate dehydrogenase and cytochrome c
are not labeled. The only proteins that have been shown to be synthesized
by the mitochondrion in vitro are the mitochondrial structural protein (4)
and an oligomyosin sensitive ATPase (43). Similarly Kadenbach (4)
confirmed these observations by demonstrating that 65% of the radioactive
amino acids incorporated into rat liver mitochondria in vitro are found
in the structural protein. In contrast only 13% are found in the soluble
protein. By incubating labeled microsomes with mitochondria in the pre-
sence of an energy generating system, Kadenbach was able to demonstrate
a transfer of label from the microsomes to mitochondria. The highest
specific activity was found in the soluble protein fraction of the mito-
chondria. Thus, it appears that the soluble mitochondrial enzymes are
synthesized in the cytoplasm then transferred to the mitochondrion. In-
direct observations indicate that this is the case for cytochrome c.
For example, in pulse-chase experiments with ascites tumor cells (25)
cytochrome c behaved as the whole cell sap. The cytochrome c specific
activity exhibited a continual increase after the chase while the
specific activity of the total mitochondrial proteins decreased.

The site of the complex integration of heme and apo-cytochrome c is also unknown. Either the heme is attached at the site of protein synthesis (i.e. cytoplasmic ribosomes) or after the protein has been transported to the mitochondrion. Neither possibility has been eliminated. The enzymes concerned with heme synthesis in mammalian systems are divided between the mitochondria and the cytoplasm. For example, enzymes involved in the initial stage (δ-aminolevulinic acid synthetase) and the final stages (coproporphyrinogen II to heme) are located in the mitochondrion, whereas enzymes catalyzing the intermediate steps are found in the cytoplasm (44). From these observations it is plausible that the attachment site is in the mitochondrion. However, other experiments have indicated that heme increases the rate of hemoglobin synthesis, possibly by stimulating the release of the nascent polypeptide chains from the ribosomes (45,46,47). This, of course, may not be true for cytochrome c synthesis; but these results indicate that heme can be transported from the mitochondrion to the cytoplasm.

Multiple Forms of Cytochrome c

There is no evidence to suggest that more than one structural gene for cytochrome c exists in vertebrates (1). Multiple chromatographic forms of cytochrome c are detected in preparations from almost all organisms but are attributed to artifacts resulting from harsh extraction conditions (1). Stewart and Margoliash (48) have compared the amino acid sequence of hog skeletal muscle, liver, kidney and brain cytochrome c with that of heart and found all proteins to be identical. There is no change in cytochrome c structure, therefore, resulting from the selection of different structural genes during differentiation of these organs. The possible existence of a fetal cytochrome c as is the case, for example, for hemoglobin (93) has

never been tested. Matsubara and Smith (49), however, may have found a genetic variant of human heart cytochrome c. The amino acid sequence was determined using pooled protein isolated from several hearts. A peptide obtained in low yield (2%) was found to have a leucine substituted for methionine at residue 65.

The situation is quite different in baker's yeast. Haploid strains synthesize two distinct molecular forms of cytochrome c, termed iso-1 and iso-2 (50). The two proteins differ by thirteen amino acid residues (51) and are under separate genetic control. From fifteen mutants exhibiting reduced amounts or no cytochrome c, six nuclear genes were mapped (52,53); one of which, cy_1, has been identified as the structural gene for iso-1 (2). Mutations in this locus abolish the synthesis of iso-1 without affecting that of iso-2. Revertants which restore iso-1 synthesis have been isolated. One such revertant has been shown to have a Glu $\longrightarrow$ Tyr change in the heme peptide of iso-1. It was postulated that the cy_1 mutation introduces a chain-terminating codon. This is compatible with one-step mutations in each direction [Glu (GAA or GAG) $\longrightarrow$ chain-terminator (UAA or UAG) $\longrightarrow$ Tyr (UAU or UAC)].

Slonimski et al. (54) have postulated that iso-2 acts as a genetic regulator of iso-1. In anaerobically growing cells, the iso-2 polypeptide chain is assumed to be preformed and acting as a repressor of iso-1 synthesis. The primary event of oxygen induction of cytochrome c synthesis, then, is to induce heme synthesis. The act of combining the prosthetic group to apo- iso-2 is thought to release the inhibition and to permit de novo synthesis of iso-1. The evidence for this model is based largely on the kinetics of synthesis of the two proteins under a

variety of conditions; oxygen induction of cytochrome c synthesis causes
an immediate appearance of iso-2 followed by iso-1 at a slower rate.
This model is supported by the recent finding that acti-dione completely
inhibits oxygen induced synthesis of iso-1, whereas iso-2 synthesis is
inhibited by only 30 to 40% (55). In addition the amount of radio-
active amino acids incorporated into iso-2 after induction of synthesis
is about one-third that found in iso-1 (56). Therefore, the evidence
that apo-iso-2 exists in anaerobic cells is strong, although indirect.
However, the inhibitor role of iso-2 is somewhat doubtful since iso-2
can constitute 20 to 30% of the total cytochrome c in normal aerobic
cultures (54). This seems excessive for a protein acting solely as a
regulator.

Cytochrome c Structure

Structural and functional aspects of cytochrome c have been the
subject of intensive study largely through the efforts of Margoliash,
Smith and their co-workers (57,58). An excellent review of this work
by Schejter and Margoliash(1) has recently appeared. To date the pri-
mary structures of twenty-five cytochromes c have been determined (57)
including that of Neurospora. Heller and Smith have determined the
complete amino acid sequence of the protein extracted from poky (59).
Like the yeast and moth proteins (57) and differing from all vertebrate
cytochromes (57), the N-terminal amino acid is unacetylated but contains
instead an additional four residues. The histidine at residue 26 is
replaced by glutamine thereby eliminating this residue as being involved
in hemochrome formation. Neurospora cytochrome c may be classified as
a "mammalian-type" cytochrome c since it will react with cytochrome

oxidase prepared from horse heart muscle (60).

Margoliash and Smith have compared the primary sequences of the cytochromes c from various species for homology (57,58). Of the conserved residues the longest stretch is the segment extending from residue 70 to residue 80:

Asn-Pro-Lys-Lys-Tyr-Ile-Pro-Gly-Thr-Lys-Met.
 70   80

This sequence has been strictly maintained throughout the whole phylogenetic scale ranging from molds to humans. Margoliash (57) has considered this behavior to indicate an integration with a structure that also has remained invariant throughout evolution. It was considered likely that this polypeptide sequence is in close proximity to the cytochrome c heme (57). This assumption is substantiated by other recent chemical studies that imply the methionine at the carboxy terminus of this segment (residue 80) is involved in hemochrome formation. This evidence comes from observations with derivatives of the protein. Tsai et al. (67) obtained a carboxymethyl substituted horse heart preparation by reacting the protein with iodoacetic acid at pH 3.0. The resulting derivative had an altered spectrum indicative of a low spin state and was unreactive in the succinate oxidase system. These changes in property were paralleled by the destruction of the two methionines at positions 65 and 80. The rate of carboxymethylation was approximately that of free methionine suggesting that these two residues are located on the surface of the molecule. Tsai et al. (62) performed the same experiment with Samia cynthia (moth) cytochrome c which contains only one methionine at residue 80. Their results indicate that carboxymethylation of this particular methionine accounts for the above observed effects. Ando et al. (63) reached the

same conclusion by performing the reaction at pH 5-6. At this pH the methionine at position 65 was first carboxymethylated followed by methionine 80. The monoderivative exhibited normal properties while the dicarboxymethylated protein was altered as before. Harbury et al. (64) found that adducts of thio ethers including N-acetylmethionine with a heme octapeptide result in normal hemochrome spectra. The heme peptide contains a histidine residue corresponding to residue 18; therefore, both the histidine and methionine previously implicated as the fifth and sixth ligands of the heme iron are present in the adduct (1).

This same amino acid segment (residues 70-80) of cytochrome c has also been implicated to participate in the cytochrome oxidase reaction. Okunuki et al. (65) obtained a monotrinitrophenyl substituted preparation of beef heart cytochrome c in which the entering group modified the lysine residue in position 72 or 73, located near the amino terminus of the invariant segement. The mono-substituted TNP protein had only 50% of the activity of the native protein in the cytochrome oxidase assay although the hemochrome spectrum was not affected. Further tri-nitrophenylation resulted in a much less loss of activity.

These data are compatible with the 4Å electron density map of horse heart cytochrome c obtained by Dickerson et al.(66). As nearly as the polypeptide chain can be followed an extended chain, which bears the sixth iron ligand, runs parallel to the long axis of the molecule and perpendicular to the plane of the heme. The shape of the ligand does not fit that of a tyrosyl, tryptophanyl or histidyl side chain. Tentatively, this ligand has been assigned to the carboxy-terminal portion of the peptide chain and could easily be the methionine at residue 80.

Purpose of the Present Research

The major purpose of this research was to study cytochrome c synthesis in poky. Particular emphasis was placed on the fact that Neurospora synthesizes twocytochromes c, C_I and C_{II}, and relationship between these two proteins; the kinetics of synthesis of C_I and C_{II} in poky as compared to wild type and other respiration deficient mutants of Neurospora; and the fact that only one cytochrome c, C_I, is found in the mitochondrion. The poky mutant of Neurospora was selected for this work because it accumulates cytochrome c and because much of the cytochrome is not bound to any particulate fraction of the cell. The goal of this research was to gather information useful to understanding mitochondrial biosynthesis in terms of individual proteins that are integrated into its structure.

MATERIALS AND METHODS

Strains of Neurospora

Neurospora crassa strains, po-6a, pof-2, mi-3-2543-2A, C-115-R4, EM-3297-2a and 4A were used in this study. EM-3297-2a and 4A are wild type strains. Po-6a carries a maternally inherited mutation called poky (10). This isolate was recovered as a single ascospore from a cross of po-3627-2a and 4A. Conidia from the original poky strain, po-3627-2a, provided by Mary B. Mitchell, aggregated causing variable germination and growth in liquid medium. The "clumping" character of the conidia segregated 1:1 in 22 randomly tested ascospores. Pof-2 carries a chromosomal suppressor of poky (14). This strain was reisolated from a cross of po-6a and pof-3627-4A for the same reason as the po-6a strain. Mi-2543-2A carries a maternally inherited mutation (11) while C-115-R4 carries a spontaneous chromosomal mutation (11).

Culture of Neurospora

Stock cultures of various strains of Neurospora were maintained on agar slants of Horowitz complete medium (67). Large amounts of conidia were grown in 1 l. wide-mouth Erlenmeyer flasks containing 200 ml. of Horowitz complete medium plus 1.5% agar (the sides of the flask were coated with a thin film of medium). Under constant illumination (68) at 25°C, abundant growth and conidiation were obtained in 7 days for poky and in 5 days for wild type.

Conidia, for inoculation of liquid cultures, were harvested from the 1 l. flasks by suspending the spores in distilled water with gentle agitation then filtered through glass wool. Spore counts were made with

with the aid of a Bright Line Haemocytometer.

Small liquid cultures of mold were grown at 25°C on a reciprocal shaker in 125 ml. Erlenmeyer flasks containing 20 ml. of Vogel's medium (87) and 2% sucrose. These were used throughout the chloramphenicol experiments. The appropriate amount of chloramphenicol (Parke, Davis and Company) was added as a solid to the sterilized medium. Large liquid cultures (16 l. of Vogel's medium plus 2% sucrose) were grown in 5 gallon Pyrex carboys as described by Hardesty (7). Sterile air was supplied at the rate of 12 l./min. after successive filtrations through Kirby and sterile glass wool filters then through sterile distilled water. This last filtration was necessary to saturate the air with water in order to alleviate the evaporation of the medium. Immediately after terminating aeration, the mold was collected by filtering the medium through 3 to 4 layers of Miracloth (Chicopee Manufacturing Company). The excess medium was removed by washing with distilled water. After squeezing out as much water as possible, the pad of mycelium was used immediately or wrapped in aluminum foil and stored at -27°C.

All cultures grown in 5 gal. carboys were inoculated with 10^7 conidia per l. of medium unless otherwise specified. Under these conditions poky cultures remained in logarithmic growth for 30 hrs. as compared to 10 hrs. for cultures inoculated with 5×10^8 conidia per l. of medium (Fig. 1).

Isolation of Mitochondria

Mitochondria were isolated according to the procedure of Luck (6). Samples of mycelia were ground immediately after harvesting from growth flasks. Mitochondrial bands were removed from sucrose gradients by means

Figure 1. Dry mass increase of _poky_ cultures started with two different
inoculums of conidia. The cultures were grown in 5 gal. carboys con-
taining 16 l. of Vogel's medium N plus 2% sucrose. To obtain the growth
represented by curve A, the culture was inoculated with 8×10^9 conidia.
The culture represented by curve B was started with an inoculum of
1.6×10^8 conidia.
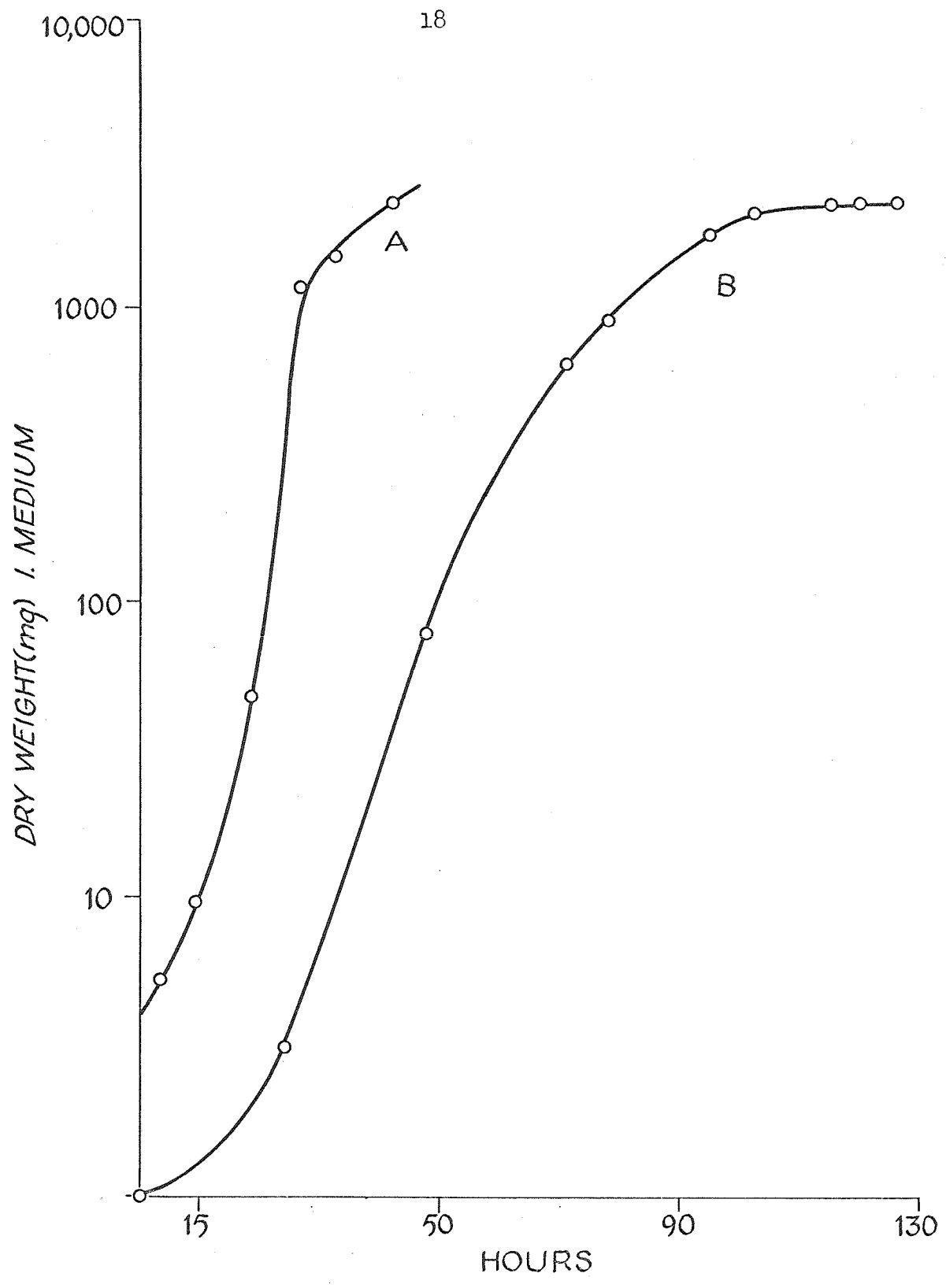
of a Pasteur pipette rather than the usual method of collecting drops through a pinhole in the base of the centrifuge tube.

Cytochrome c Purification

In these investigations two methods were employed for isolating Neurospora cytochrome. A small scale purification procedure (less than 50 g. of Neurospora) was evolved through several modifications in the early stages of this work. The final form of this scheme incorporates the ammonium hydroxide extraction step described by Hardesty (7) and the use of Amberlite CG-50 as described by Hagihara et al. (69) for Amberlite XE-64. The buffers and elution gradient for the Amberlite CG-50 columns are those described by Sels et al.(50).

The small scale purification procedure was modified somewhat to accommodate larger quantities of mold more efficiently. A Waring blender was substituted for a mortar in grinding the mycelial pad. Also acid-washed Hyflo-Super Cel employed by Hardesty (7) was substituted for Amberlite CG-50 to concentrate the cytochrome c from crude extracts. The small scale procedure was retained nevertheless since several steps were less time consuming. Therefore both methods are described here.

Large Scale Purification. All steps were carried out at 5°C except for the Amberlite CG-50 columns which were run at room temperature.

Step 1. Pressed dry pads of Neurospora (60-500 g.) were frozen by dipping in liquid nitrogen. They were then ground in a metal Waring blender until a fine powder resulted (1-2 min.). After carefully pouring liquid nitrogen over the mycelial powder, it was ground a second time for 1 min. then allowed to thaw in the cold.

Step 2. The ground mycelium was mixed with 2 volumes of 0.05 $\underline{M}$ Tris buffer pH 8.6. The pH was brought to 10.5 with concentrated ammonium hydroxide and vigorous stirring. After sitting for 15 min. the stirring was started again as the pH was reduced to 8.0 with glacial acetic acid. The homogenate was transferred to 250 ml. plastic cups and centrifuged at 3,000 x g for 10 min. in a Servall R-2 centrifuge. The precipitate was washed 3 times with buffer and the washes were combined with the original supernatant.

Step 3. The red supernatant was centrifuged at 79,000 x g in a Spinco Model L preparative centrifuge for 60 min. The red, opaque precipitate yielded little cytochrome c on extraction with ammonia. It was routinely discarded. At this stage the preparation was a clear, red solution. Dialysis was carried out overnight against 100 volumes of 0.005 $\underline{M}$ phosphate buffer, pH 7.0, containing 10^{-4} M $K_3Fe(CN)_6$. Occasionally the cytochrome c solution became cloudy but was cleared by centrifugation at 10,000 x g for 10 min.

Step 4. The dialyzed supernatant was passed slowly through acid washed Hyflo-Super Cel (7) packed on a coarse fritted glass funnel (10 cm. in diameter). Suction was employed to maintain a constant flow rate. The absorbed cytochrome c was washed with several volumes of distilled water or until the eluate was clear. Hyflo-Super Cel containing the absorbed cytochrome c was then poured into a column, and the cytochrome c was eluted with 0.5 $\underline{M}$ NaCl in 0.05 $\underline{M}$ phosphate buffer (pH 7.0). Dialysis was carried out as described in Step 3.

Step 5. A 1.5 x 90 cm. column of Amberlite CG-50 treated by the hypochloride method of Hagihara et al. (69) was equilibrated with 0.05 $\underline{M}$

phosphate buffer pH 7.0. The dialyzed cytochrome c solution from the previous step was placed on the column. The cytochrome c was absorbed as a narrow red band at the top of the resin. Chromatography was carried out at a flowrate of 5.5 ml. per hour using a linear gradient with respect to ionic strength preformed between 150 ml. of 0.05 $\underline{M}$ phosphate buffer pH 7.0 and 0.05 $\underline{M}$ phosphate buffer pH 7.0 plus 0.5 $\underline{M}$ NaCl. Both buffers contained 10^{-4} $\underline{M}$ $K_3Fe(CN)_6$. Fractions of 2 ml. were collected. Tubes of cytochromes C_I and C_{II} were pooled separately and dialyzed as in Step 3.

Step 6. After dialysis, the pools of cytochromes C_I and C_{II} were rechromatographed separately on 1.5 cm x 50 cm. columns of Amberlite CG-50 under the same conditions as in Step 5. Contents of the appropriate fractions were pooled, dialyzed against the appropriate buffer or distilled water and then frozen at -20°C.

Small Scale Purification. Step 1. Mycelial pads of Neurospora weighing less than 50 g. were frozen in liquid nitrogen and then ground in a mortar three times. Liquid nitrogen was poured over the tissue between each grinding.

Steps 2 and 3. These were carried out as described for large scale purification.

Step 4. The dialyzed supernatant from the high speed centrifugation was passed through 16 ml. of Amberlite CG-50 packed in a 2 x 28 cm. column. The absorbed cytochrome c was washed by removing the resin from the column, suspending it in distilled water and then centrifuging at 3,000 x g for 5 min. The washing was repeated until the supernatant was clear (3-4 times). The cytochrome c was eluted with 0.5 $\underline{M}$ NaCl in 0.05 $\underline{M}$ phosphate buffer after the resin was repacked in the column.

Steps 5 and 6. These steps were carried out as described for large scale preparations except that 1 x 10 cm. columns were used and 1 ml. fractions were collected.

Spectra

Cytochrome c concentrations of column fractions were routinely determined by scanning the 590-500 mμ region of the visible spectrum with a Cary Model 15 recording spectrophotometer equipped with a repetitive scan apparatus. Samples were reduced prior to scanning by adding a few grains of sodium dithionite. A molar extinction coefficient of 29,000 at 550 mμ was assumed (70). Ultraviolet spectra were obtained before the addition of sodium dithionite to the sample.

Cytochrome c was determined by different spectra in cell extracts and mitochondria by procedure of Williams (71).

The visible absorption spectrum of the oxidized form of pure cytochromes C_I and C_{II} in 0.05 $\underline{M}$ phosphate buffer, pH 7.0, was determined after adding $K_3Fe(CN)_6$ to $10^{-4}\underline{M}$. After dialysis against the same buffer, the proteins were reduced with sodium dithionite and the visible region was recorded.

Sedimentation Constants of C_I and C_{II}

Sedimentation constants of cytochromes C_I and C_{II} were determined by the band centrifugation technique of Vinograd et al. (72). Pooled fractions of each of the two cytochromes c from the final Amberlite CG-50 column of the purification procedure were dialyzed overnight against 0.05 M Tris buffer (pH 7.6). Each fraction in dialysis tubing was packed in dry Sephadex G-25 at 4°C (73). After a three-fold reduction in volume the samples were exhaustively dialyzed against the same

buffer. The final concentration of each cytochrome c was 10 mg. per mL.
The centrifugation was performed in Spinco Model E ultracentrifuge
equipped with ultraviolet absorption optics. A standard cell equipped
with sapphire windows and a 12 mm. Kel-F band type III centerpiece was
employed. After reaching a speed of 63,650 r.p.m. the first photograph
was taken at 4 min.; the remainder were taken at 16 min. intervals. A
record of protein concentration versus radius was obtained from the de-
veloped film with a Joyce-Loebl double beam microdensitometer.

Pulse-Labeling of Neurospora Proteins

After harvesting, pads of Neurospora were washed with distilled
water. The excess liquid was removed by pressing the mycelial mat be-
tween paper towels or on a Buchner funnel with suction. The pad was
resuspended with gentle agitation in 20 volumes of sterile Vogel's
medium N plus 2% sucrose. This ratio of buffer to mycelium made a
thick slurry when the mold was evenly suspended. Usually from 1 to
50 μc of H^3- or C^{14}-lysine was added to each sample ranging in weight
from 20 to 200 g. During the pulse, the incubation mixture was gently
shaken on a reciprocal shaker. The incorporation was stopped by collecting
the mold on a Buchner funnel with suction. After thoroughly washing with
distilled water to remove the radioactive medium, the tissue was ground
immediately or frozen at -20°C.

Fractionation of Neurospora Labeled with C^{14} Amino Acids

The distribution of C^{14} in Neurospora proteins pulsed with C^{14}
amino acids was determined by the fractionation procedure of Roberts
et al. (74). Mycelial pads, 100 to 120 mg., were suspended in 20 mL.
of sterile Vogel's medium N plus 2% sucrose. Approximately 0.5 μc of

C^{14} were added to each sample. After shaking for 15 min. at 25°C, the tissue was collected on a Buchner funnel then washed with distilled water. The cells were immediately suspended in cold 5% trichloroacetic acid. The extraction procedure was always carried to completion the same day.

Radioactivity Determinations

Counting of Column Fractions   For counting H^3- or C^{14}-labeled cytochrome c, 0.2 ml. aliquots were removed from every other fraction from the final Amberlite CG-50 column and put into glass scintillation vials filled with Bray's solution (75). The samples were counted in a Nuclear Chicago 720 Series scintillation counter by the channels ratio method (76). The machine settings and a quenching curve were determined with standards purchased from Nuclear Chicago. The effect of cytochrome c color on the counting efficiency was determined by adding varying amounts of the protein to known amounts of radioactive amino acids. The efficiency of counting was calculated before and after the addition of cytochrome c. All reported values are corrected to 60% counting efficiency for C^{14} and 20% counting efficiency for H^3.

Counting of H^3 Labeled Amino Acids from Peptide Hydrolysates

For the determination of H^3 in amino acids separated electrophoretically on paper and stained with the cadimum-ninhydrin reagent (see Table 1) the following procedure was used: the methanol extract of the paper was transferred to scintillation vials after reading optical densities, then two drops of concentrated HCl were added to each vial to bleach the red color. The methanol-HCl solution was evaporated with gentle heating after which 0.15 ml. of Hyamine Hydroxide (Nuclear Chicago)

was added to neutralize any remaining HCl and to dissolve the residue. The vials were filled with Bray's solution and counted by the channels ratio method.

Counting of Fractions from Robert's et al. Procedure (74) _Neurospora_ labeled with C^{14} amino acids was extracted by the procedure of Roberts _et al._ (74) to determine the distribution of radioactivity in the cell. Aliquots (usually 0.02 ml.) of the resulting fractions were pipetted on aluminum planchets except for the NaOH fraction which was pipetted on copper planchets. Acetone was added to each planchet to spread the sample. After drying under a heat lamp the samples were counted by a Nuclear Chicago gas-flow low background counter. Background was determined by counting empty planchet holders.

Tryptic Peptide Maps

Cytochromes C_I and C_{II} (1 mg. each) in 0.2 N NH_4HCO_3 were heated for 60 sec. in a boiling water bath. Each sample of denatured protein was digested for 7.5 hrs. with 3% trypsin (TPCK treated) then applied to Whatman No. 3 chromatographic paper. Chromatography-electrophoresis was carried out essentially as described by Katz _et al._ (77). Chromatography was performed for 24 hrs. and electrophoresis for 1 hr. at 55.5 volts per cm. After drying for 20 min. at 80°C the papers were stained with the collidine ninhydrin reagent (see Table 1). Polaroid 55/PN film 4 x 5 inches was used to photograph the papers.

Cleavage with Cyanogen Bromide

Cleavage with cyanogen bromide was carried out with both cytochromes C_I and C_{II} as described by Heller and Smith (59). The reaction products

were separated on a 1.5 x 60 cm. Sephadex G-50 column equilibrated with 10%
acetic acid. Aliquots (0.1 ml.) from every second tube were analyzed by the
ninhydrin method after alkaline hydrolysis (81). Fractions from ninhydrin
positive peaks were pooled, lyophilyzed and then dissolved in 100 μl. of
distilled water. Each peptide was further purified by chromatography
on Whatman No. 3 paper in butanol: acetic acid: water (4:1:5) for 16 hrs.
Peptides were located with the ninhydrin detection spray (see Table 1).
The peptides were eluted from the paper with constant boiling HCl into
0.5 x 7 cm. Pyrex tubes and then hydrolyzed under vacuum. Amino acid
analysis was carried out by paper electrophoresis.

Enzymatic Digestion

Digestion of Cytochrome c with Trypsin and Chymotrypsin Cytochrome
c was denatured with 70% alcohol as described by Matsubara and Smith (49).
The denatured cytochrome c was precipitated by centrifugation at top
speed in a clinical centrifuge then redissolved in 5.0 ml. of distilled
water and adjusted to pH 8.0 with 0.005 N NaOH. The digestion was per-
formed at room temperature with 2% TPCK treated trypsin (78) (Worthing-
ton, twice crystallized) or 2% chymotrypsin (Worthington, twice crys-
tallized). The digestion mixtures were maintained at pH 8.0 with
0.0509 N NaOH by automatic titration in a Radiometer TTTI pH-stat.
After 12 hrs. an additional 2% trypsin or chymotrypsin was added. The
reaction was terminated after 24 hrs. by freeze-drying.

Peptide Digestion with Pronase Peptide C_I-11 (80 mμ moles) was
dissolved in 0.02 ml. of water and adjusted to pH 6.5 with 1 M ammonium
hydroxide. The digestion was carried out for 24 hrs. at 35°C with 0.05

mg. of pronase (Cal Biochem) (79). The reaction was terminated by freeze-drying.

Column Chromatographic Separation of Tryptic and Chromotryptic Peptides

Dowex 50-X2 specially blended for peptide separation (Proj. 449) was obtained from Beckman Instruments Co. After regeneration by the procedure of Moore and Stein (80) the resin was equilibrated with 0.2N pyridine-acetate buffer pH 3.1. The chymotryptic or tryptic digestion mixture was chromatographed on a 1 x 34 cm. column at 40°C at a flow-rate of 17 ml. per hr. maintained by a constant delivery pump. Fractions of 2 ml. were collected. The buffer systems were those described by Matsubara and Smith (49). After the addition of the sample to the column the elution was begun with 160 ml. of 0.2N pyridine-acetate buffer pH 3.1. The majority of the peptides were eluted by a gradient with respect to ionic strength and pH. This was prepared by placing 200 ml. of 0.2N pyridine-acetate, pH 3.1, in the mixing chamber and 200 ml. of 0.2N pyridine-acetate, pH 5.0, in the reservoir. Any remaining peptides were eluted by an additional 160 ml. of 2.0 N buffer followed by 100 ml. of 4.0 N pyridine-acetate, pH 5.6.

From every third fraction 0.2 ml. was removed for ninhydrin analysis after alkaline hydrolysis (81). Contents of tubes from nin-hydrin positive peaks were pooled, lyophilyzed and stored at -27°C in 1.0 ml. of distilled water.

Further Purification of Peptides

The purity of each peptide fraction (10-20 μl.) was examined by electrophoresis on Whatman No. 3 paper at two different pH values. The

conditions were: pH 3.5, 4.5 hrs. at 75 volts/cm. and pH 6.0, 8.0

hrs. at 26.7 volts/cm. The compositions of these buffers are as follows:

pH 3.5, 1% pyridine-10% acetic acid; pH 6.0, 20% pyridine - 2.5% acetic

acid. In some cases preparative peptide maps were employed. Chromato-

graphy (butanol: acetic acid: water (4:1:5) for 16 hrs.) was followed

by pH 3.5 electrophoresis. The papers were dried 20 to 30 min. in an

80°C oven. They were then dipped in collidine-ninhydrin reagent (82) and

developed for 10 min at 80°C. After noting the color of each peptide the

paper was photographed.

After these tests one half of each peptide fraction from the column

was lyophilyzed then dissolved in 30 μl. of distilled water and applied

to Whatman No. 3 paper with a Beckman sample applicator (catalog no.

320005). By the appropriate electrophoresis conditions the peptides of

each pool were separated. Peptides were located by spraying lightly

with ninhydrin detection reagent followed by heating for 10 min. at

80°C. Each peptide was eluted from the paper with either constant boil-

ing HCl or distilled water in a chamber whose atmosphere was saturated

with the solvent. Usually one half of the eluted peptide, in constant

boiling HCl, was transferred to a thick-wall Pyrex tube (0.5 x 7cm.).

This was flushed with nitrogen and sealed under pressure. Hydrolysis

for 24 hrs. at 105°C was sufficient except for peptides containing an

Ile-Ile sequence. These were hydrolyzed for 48 hrs.

Performic Acid Oxidation

Performic acid oxidation of the tryptic heme peptide of C_{II} was

carried out according to the procedure of Hirs (83). A 12 fold molar

excess of performic acid was allowed to react with the peptide for

2.5 hrs. at -10°C. The reaction was terminated by freeze-drying.

Amino Acid Analysis

Analysis on Paper   Each acid hydrolyzed peptide was dried in vacuo
over KOH pellets and then dissolved in 50 μl. of distilled water.
Usually 10-20 μl. were sufficient for analysis. Samples were spotted
on Whatman No. 3MM (2 x 0.46m.) with a Beckman sample applicator (cata-
log no. 320005). Electrophoresis at 40 volts/cm. for 1.75 hrs. (pH 1.9) at
42-45°C in a Gilson Model DW Electrophorator separated all amino acids
except the combinations of tyrosine-aspartic acid and proline-threonine
which migrated together. After drying for 30 min. at 80°C the paper
was dipped in the cadmium-ninhydrin reagent (Table 1) which gives a
deep red color for all amino acids except proline. Proline develops as
a light yellow color. The color development was carried out overnight
in the dark and in an ammonia free atmosphere. Standard amino acid
mixtures were run along side the samples for identification purposes and
to calibrate the cadmium-ninhydrin color. Each ninhydrin positive spot
was shredded to fit into a 1 x 7.5 cm. test tube. The color was eluted
with 3.0 ml. of absolute methanol by shaking for 1.5 hrs. at 25° (84).
Optical densities were determined at 500mμ. Amino acid concentrations
were calculated from standard curves.

For analysis of tyrosine and aspartic acid, amino acids were
separated by chromatography-electrophoresis. Butanol-acetic acid-water
(4:1:5) descending chromatography was performed for 4 hrs. or until the
phenol red dye (77) had moved 14 cm. Electrophoresis was carried out

at pH 1.9 as described above. For the detection of proline the cadmium-isatin reagent (see Table 1) was used. Electrophoresis (pH 1.9) of acid-hydrolyzed peptides suspected of containing proline was run in duplicate. One sample was dipped in the cadmium-ninhydrin reagent to determine the concentration of all amino acids except proline. The second sample was developed with the cadmium-isatin reagent (84). Electrophoresis of known concentrations of threonine and proline and proline alone was also carried out. These were developed with the cadmium-isatin reagent for the purpose of calculating standard curves for proline and proline in the presence of threonine. The presence of threonine was found to have no effect on the determination of proline by the cadmium-isatin reagent.

Column analysis The method employed for automatic amino acid analysis was either a microversion of the procedures described by Piez and Morris (85) or the method of Spackman, Stein and Moore (86).

TABLE 1

COLOR REAGENTS FOR PEPTIDE AND AMINO DETECTION

Ninhydrin Peptide Detection Reagent

Ninhydrin	50mg.
Absolute Ethanol	75ml.
2N Acetic Acid	25ml.

Ehrlich's Reagent for Tryptophan (82)

p- Dimethylaminobenzaldehyde	1g.
Acetone	90ml.
Concentrated HCl	10ml.

Collidine-ninhydrin Reagent (82)

Absolute ethanol	600ml.
Glacial Acetic Acid	200ml.
Collidine	80ml.
Ninhydrin	1g.

Cadmium-ninhydrin Reagent (84)

Stock solution:	Cadimum Acetate	1.0g.
	H_2O	100ml.
	Glacial Acetic Acid	20ml.

To 24 ml. of stock solution was added 200ml. acetone and 2.0 g ninhydrin.

Cadmium-isatin Reagent (84)

Stock solution:	Cadimum Acetate	10g.
	H_2O	90ml.
	Glacial Acetic Acid	10ml.
	Propan-2-OL	950ml.

To 25.5 ml. of stock solution was added 0.25 g. isatin.

RESULTS

Purification The preparation of mammalian-type cytochromes c is
relatively simple because of their low molecular weight, basicity and
relative stability to harsh conditions (1). Purification procedures
for cytochrome c follow a general pattern based on these properties.
The critical step in the procedure is the extraction of the protein.
Since cytochrome c is membrane bound, harsh conditions such as acid,
base, or organic solvents are necessary to render it extractable (1).
The efficient absorption of cytochrome c by cation exchanges has been
employed to absorb the protein from crude tissue extracts (1). Absorp-
tion on exchange resins affords a rapid method of concentrating cytochrome
c since it is eluted by salt (0.5 to 1.0 g./l.) as a concentrated band
that is usually 50-70% pure. Ammonium sulfate fractionation followed by
repeated column chromatography with cation exchange resins results in
a pure, native cytochrome c free from preparational artifacts (1).

The purification procedure in the present study generally follows
this outline. The cytochrome c was extracted at pH 10.5 by titrating
the cell homogenate with ammonium hydroxide followed by low speed cen-
trifugation to remove cellular debris. The choice of a next step was
found to be critical since cytochrome c is destroyed in crude poky ex-
tracts unless a relative high pH is maintained to inhibit the action of
proteolytic enzymes (7). Both treatment with 70% ammonium sulfate as
described by Hardesty (7) and high speed centrifugation (79,000 x g. for
one hr.) remove the proteolytic activity. In preliminary experiments
the base-extracted homogenate was treated with 70% ammonium sulfate.
This step was discarded as being time consuming and awkward due to

exhaustive dialysis necessary to remove the salt. High speed centrifugation, on the other hand, circumvented both disadvantages and was therefore routinely employed. After dialysis, cytochrome c could be readily concentrated from the high speed supernatant by absorption on Hyflo-Super Cel. This material could be washed with large volumes of water without detectable losses of the protein. Yet the cytochrome c was quantitatively recovered by elution with high salt (either 0.5 M NaCl or 70% $(NH_4)_2SO_4$).

As a next step, column chromatography was carried out on Amberlite CG-50. Initially, CM Sephadex 25 was employed, but it was discarded. Shrinkage of the CM Sephadex 25 resin during gradient elution caused reduced flow rates and variable separations; Amberlite CG-50, however, does not have these disadvantages.

The ratio of the optical densities of the reduced α-band maximum and protein bands ($A_{550m\mu}$ reduced/$A_{280m\mu}$ oxidized) is a rough indication of cytochrome c purity (1). Spectra were routinely determined after each purification step. By this criterion the Hyflo-Super Cel step effects a 200 fold purification (Table 2) and yields 70% pure cytochrome c. After chromatography on Amberlite CG-50 the 550/280 ratio ranged from 1.20 to 1.26. It was assumed that this was pure cytochrome c. These values are in good agreement with the value of 1.25 reported by Heller and Smith for pure Neurospora cytochrome c (59).

Chromatographic Separation of C_I and C_{II} When preparations of cytochrome c extracted from log phase cultures of poky are chromatographed on Amberlite CG-50 or CM-Sephadex 25, two cytochromes c are eluted (Fig. 2a). The two peaks are designated cytochrome C_I and cytochrome C_{II} (based on

TABLE 2

PURIFICATION SUMMARY FOR CYTOCHROME C

Purification Step	Optical Density at 550 mμ (reduced) / Optical Density at 280 mμ (oxidized)
High Speed Supernatant	0.005
Hyflo Super Cel	1.165
Amberlite CG-50	1.200

At each purification step two 0.2 ml. samples were removed for spectra. $K_3Fe(CN)_6$ was added to a final concentration of 10^{-4} $\underline{M}$ to one sample and the ultraviolet region of the spectrum was recorded with a Cary Model 15 recording spectrophotometer with a solution of 10^{-4} $\underline{M}$ $K_3Fe(CN)_6$ in the reference cell. A recording of the visible spectrum of the second sample was taken after adding a few grains of sodium dithionite. Distilled water was used as the reference solution. The ratio of the optical density of the reduced sample at 550 mμ and the optical density of the oxidized sample at 280 mμ was routinely employed as a crude indication of cytochrome c purity. Cytochrome c exhibiting a ratio of 1.20 to 1.26 was considered to be pure.

the order of elution) and hereafter are referred to simply as C_I and

C_{II}. The two proteins elute at 0.275 $\underline{M}$ and 0.290 $\underline{M}$ sodium ion re-

spectively, which is the same cation concentration range for the elu-

tion of native horse heart cytochrome c from Amberlite IRC-50 (1).

The separation of C_I and C_{II} is highly dependent on the flow rate of

the column. For example, at a flowrate of 5.5 ml. per hour, both

proteins elute as sharp, distinct bands (Fig. 2a); but at 10 ml. per

hour the cytochrome c elutes as a broad band, and as a result the peaks

of C_I and C_{II} overlap.

To test whether the two cytochromes c are stable chromatographic

species, fractions of C_I and C_{II} from the column in Fig. 2a were pooled

separately, then rechromatographed (Figs. 2b and 2c). Both proteins

eluted as single peaks at the same cation concentrations as in the column

shown in Fig. 2a. A slight shoulder on the leading edge of the C_{II} peak

is the result of contaminating C_I. Furthermore, when fractions contain-

ing C_I and C_{II} from the columns in Figs. 2b and 2c were pooled and then

rechromatographed on the same column, an elution profile identical to

that of Fig. 2a was obtained. Therefore C_I and C_{II} were considered not

to be interchangable on ion exchange columns.

Multiple chromatographic forms of cytochrome c have been found in

cytochrome c preparations from almost all sources (1). Two of these,

the oxidized and reduced forms of the protein, are naturally occurring.

However, all other forms have been attributed to artifacts of prepara-

tion (1) with the exception of the iso-cytochromes from yeast (54).

These artifacts can be conveniently grouped into three classes: a)

polymers, b) deamidated monomers, and c) aggregates with basic proteins.

Figure 2. Chromatographic separation of C_I and C_{II}. Cytochrome c was extracted from a 40 hr. sample of poky then purified (Methods). a. Primary chromatography of the cytochrome c on a 1 x 10 cm. Amberlite CG-50 column. b. Rechromatography of C_{II} obtained by pooling the appropriate fractions (39-43) from the column in a. c. Rechromatography of C_I obtained by pooling fractions 34-38 from the column in a.
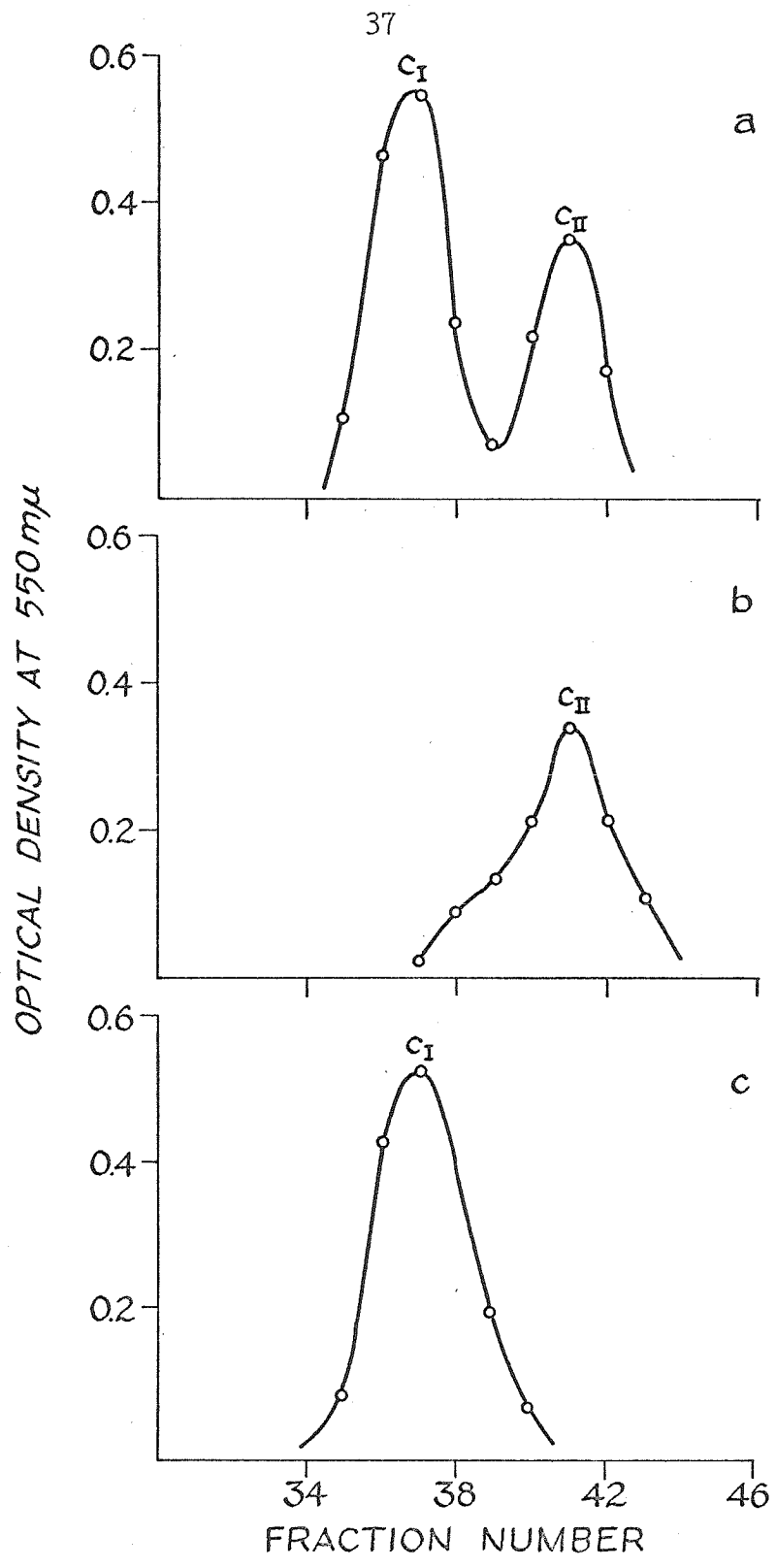
Experiments were undertaken to preclude the possibility that the chromatographic separation of C_I and C_{II} is due to an artifact of one of the above classes. C_I was initially considered to be "native" cytochrome c since it had identical elution characteristics with a sample of "native" poky cytochrome c provided by Drs. Heller and Smith.

Oxidation State   The oxidized and reduced forms of poky cytochrome c will separate on Amberlite CG-50 as shown in Fig. 3. A partially oxidized sample was chromatographed under standard conditions (Methods) except that $K_3Fe(CN)_6$ was omitted from the gradient buffers. Three peaks of cytochrome c are eluted. The 600 to 500 mμ region of the visible spectrum of all cytochrome c containing fractions was scanned before and after reduction with sodium dithionite. This was done to ascertain the oxidation state of the protein in the various peaks. From the ratio of the optical densities at 550 mμ of the reduced and untreated samples, it is obvious that peak I is reduced cytochrome c, and that the cytochromes c in peaks 2 and 3 are in the oxidized state. The cytochromes c from peaks 2 and 3 were found to be C_I and C_{II}, respectively, by rechromatography. However, the cytochrome c from peak I was not rechromatographed; therefore, it is not known whether this is only one of the two proteins in the reduced state or a mixture of both. It can be concluded, nevertheless, that the separation of C_I and C_{II} on Amberlite CG-50 does not result from a difference of oxidation state.

Polymers   Polymeric forms of cytochrome c bind more tightly to ion exchange resins than monomeric forms (88). Since C_{II} elutes more slowly than C_I from Amberlite CG-50, the possibility existed that C_{II} was a polymeric form of C_I. To test this, the sedimentation coefficients

Figure 3. Chromatographic separation of the oxidized and reduced forms

of <u>Neurospora</u> cytochrome c. A sample of partially oxidized <u>poky</u> cyto-

chrome c was chromatographed on a 1.5 x 43 cm. column of Amberlite CG-50

under the usual conditions (Methods) except that $K_3Fe(CN)_6$ was omitted

from the gradient buffers. Spectra of the eluted cytochromes c were

determined before and after reduction with sodium dithionite. The

elution profile is plotted as the optical density of the α-band of

cytochrome c (550mμ) after reduction.
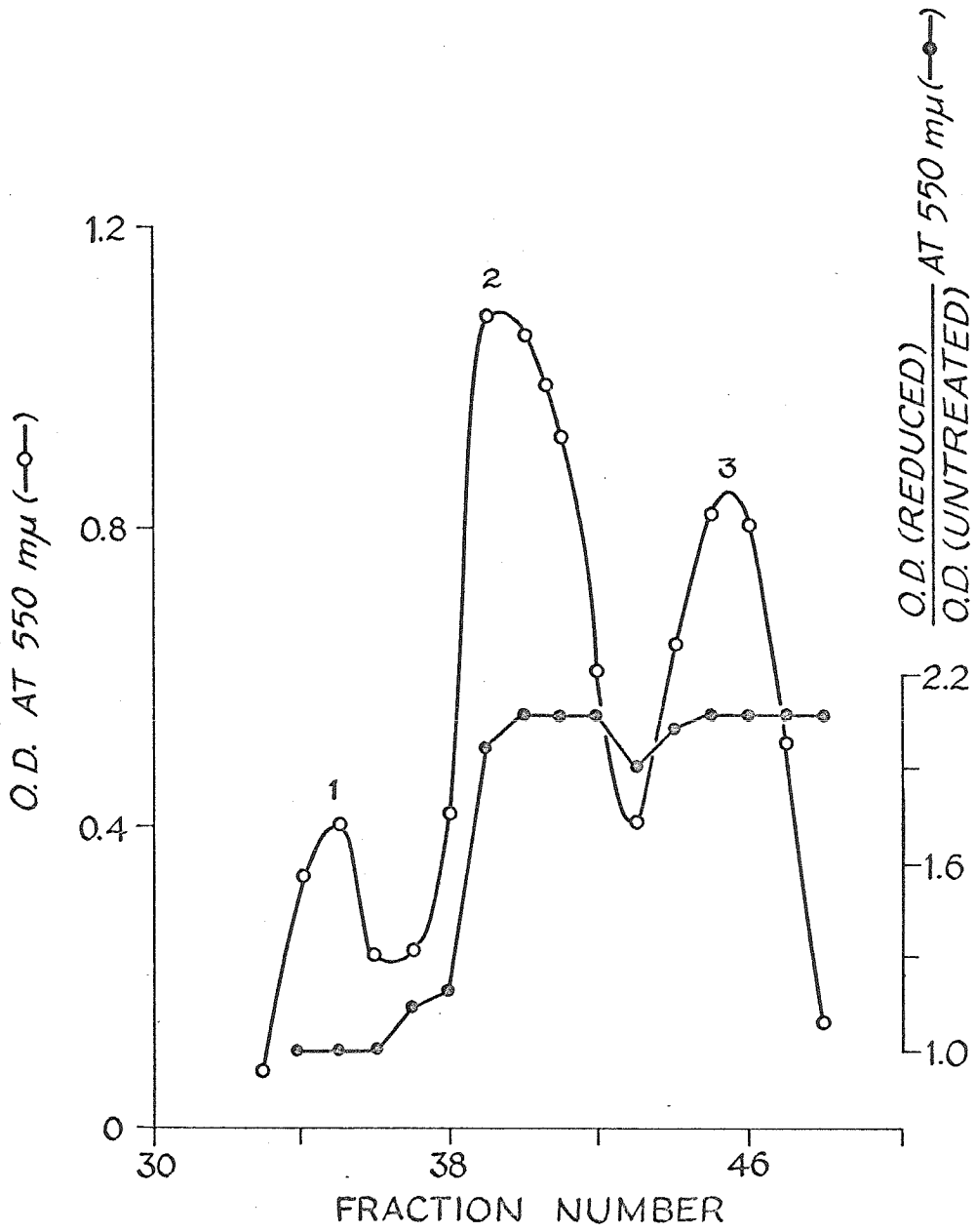
of C_I and C_{II} were determined by the band centrifugation technique (72).

In this experiment, the lamella consisted of 25 μl. of 10 mg./ml. of

cytochrome c in 0.05 $\underline{M}$ Tris pH 7.6 and the bulk solvent was 1 $\underline{M}$ NaCl in the

same buffer. The sedimentation coefficients were evaluated from the

motion of the band center with the equation

$$lnr = S\omega^2 t + constant$$

where r, S, ω and t are the radial distance to the band center, sedimen-

tation coefficient, angular velocity and time. Correction to water and

20°C was made by multiplying the calculated sedimentation coefficients

by the equation

$$\frac{1 - \overline{V}_{20}\, \rho_{w,20}}{1 - \overline{V}_t\, \rho_{t, \text{ solvent}}}$$

It was assumed that $\overline{V}_{20} = \overline{V}_t = 0.721$ (59). The value of $\rho_{t, \text{solvent}}$

was taken to be 1.0385 (89) and the value of $\rho_{w,20}$ to be 0.99823 (89).

The sedimentation coefficients calculated for C_I and C_{II} from a plot

of lnr versus t (Fig. 4) were found to be the similar: $S_{20,w}$ $(C_I) = 1.62$,

$S_{20,w}(C_{II}) = 1.66$.

These values are low compared to a value of 1.94 for C_I determined by

boundary sedimentation by Heller and Smith (59) and values of 2.12 and

2.20 determined by the same method for wild type and poky cytochrome c

by Hardesty (7). Two reasons can be given for this discrepancy. First,

the values in the present study were not corrected to zero cytochrome c

concentration; and second, the correction for the effect of 1 $\underline{M}$ NaCl on the

sedimentation coefficient of cytochrome c may not be accurate. The effect

of high salt concentrations on the sedimentation behavior of DNA has been

Figure 4. Band centrifugation of C_I and C_{II}. Positions of band maxima

were measured from densitometer tracings of photographs taken at the

indicated times. The logarithm of the distance (cm.) of the band

maxima from the center of rotation is plotted as a function of time.
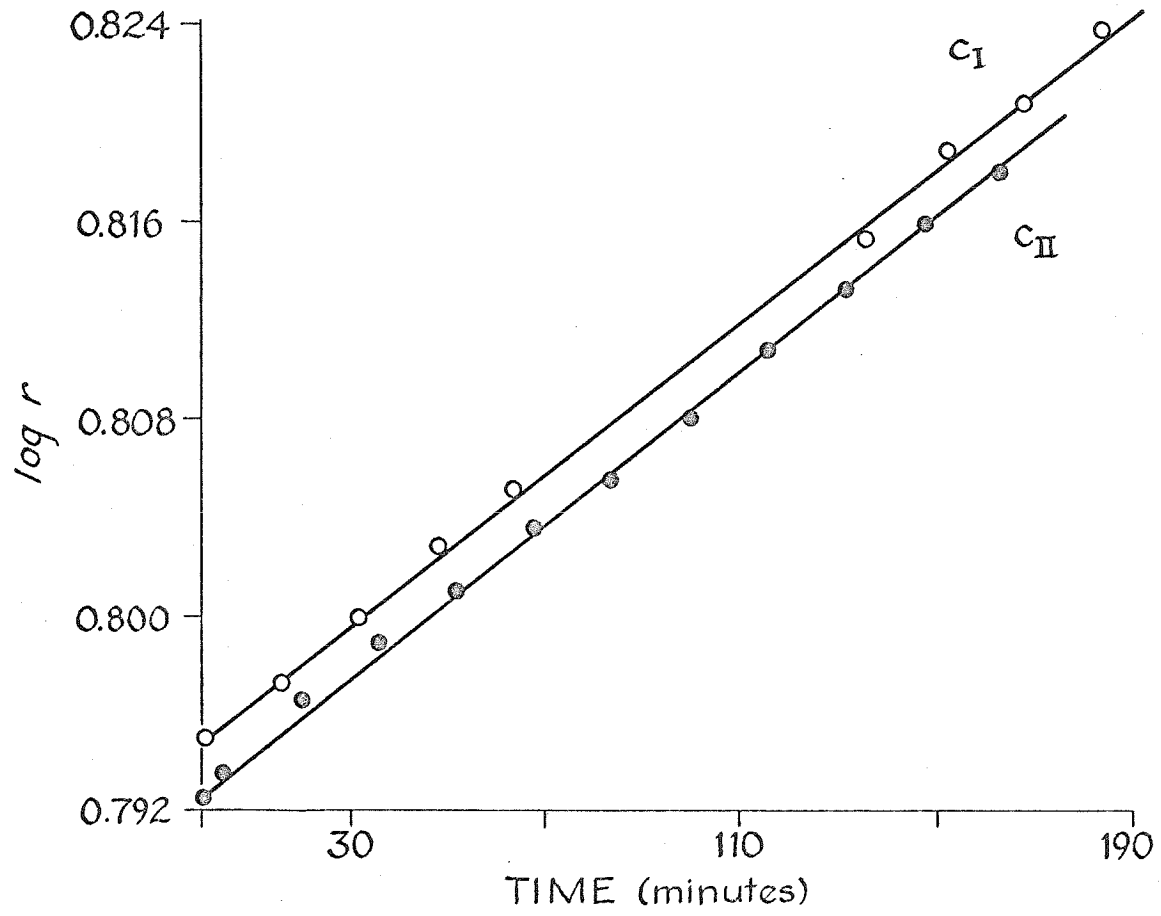
measured by Vinograd et al. (72) but this has not been extensively tested for proteins.

Deamidated Monomers Deamidated monomers of cytochrome c have been reported to elute as a shoulder on the leading side of the peak of monomeric native protein (1). By this criterion, then, C_{II} is not a deamidated form of C_I since it elutes more slowly on Amberlite CG-50 than does C_I. Occasionally, however, a shoulder was detected on the leading edge of the C_I peak. This was found not to be reduced cytochrome c and was assumed to be a deamidated artifact. One could consider this indicative that the chromatography conditions were stringent enough to separate such artifacts.

Aggregation with Basic Proteins More difficult to rule out was the possibility that C_{II} is aggregated with basic proteins. Such artifacts tend to elute more slowly than native cytochrome c and are difficult to separate chromatographically (1). The fact that C_{II} has the same sedimentation coefficient as C_I and that the ratio of optical densities at 550 mμ and 280 mμ for the pure protein is 1.2, however, argues against aggregation.

Finally, the best evidence that two cytochromes c are not artifacts resulting from harsh alkali extraction is based on a novel feature of the poky mutation. Hardesty found that 67% of the cytochrome c in young poky is not bound to particulate fractions of the cell (7). Advantage was taken of this fact by extracting cytochrome c from young poky with 0.05 M Tris buffer pH 8.6. Chromatography of the buffer extracted cytochrome c gave an elution profile similar to that of Fig. 2a; both C_I and C_{II} were detected.

Physiological Data

Controls for Radioactive Experiments   Cytochrome c has been reported
to aggregate with basic proteins (1) as previously discussed. Most of
the experiments reported here employed either H^3- or C^{14}-lysine. Since
lysine is a basic amino acid, the possibility exists that it also aggre-
gates non-specifically with cytochrome c. The following experiment was
designed to determine if lysine is removed during cytochrome c puri-
fication.

A 20 g. sample of 40 hr. poky was divided equally; to one half was
added 1 μc. of C^{14}-lysine and to the other 1 μc. C^{14}-phenylalanine.
After a 30 min. pulse, the samples were collected and cytochrome c was
purified from each. The incubation medium of both samples was checked
for radioactivity. Less than 1% of the initial radioactivity remained
in each, indicating that both amino acids were taken up equally well
by the mold. The specific activities of the purified cytochrome c were
corrected for the difference in specific activity of the radioactive
amino acids.

Assuming that cytochrome c is uniformly labeled during the 30
min. incubation and that lysine does not aggregate with the purified
protein, the ratio of the specific activities (C^{14}-lys cytochrome c/
C^{14}-phe cytochrome c) should be 3.5. This value is based on the com-
position of the protein; there are 3.5 times as many lysine residues
as phenylalanine residues in Neurospora cytochrome c (59). An experi-
mental value of 3.6 was found, indicating that free radioactive lysine
was removed during cytochrome c purification.

To determine the distribution of radioactive lysine among the various classes of molecules in poky, a sample of poky was incubated for 15 min. with C^{14}-lysine. The tissue was extracted according to the procedure of Roberts et al. (74). As shown in Table 3 the radioactivity is distributed between the small metabolite pool (50%) and the protein fraction (30%). Consistently 10 to 15% of the radioactivity was found in the nucleic acid fraction.

Synthesis and Amounts of C_I and C_{II} in Different Ages of Poky

The goal of this experiment was to determine rates of cytochrome c synthesis, total amount of cytochrome c, and amounts of C_I and C_{II} at different ages of poky.

Poky cultures were grown for 10, 20, 37, 51 and 75 hrs. These ages correspond to pre-log, early-log, log, late-log and post-log cultures respectively (see Fig. 1). Care was taken to reproduce growth conditions from culture to culture. Seven day-old conidia, grown under constant illumination (Methods), were used for inoculation. Since the growth rate of poky is strongly dependent on oxygen (90), the amount of sterile air bubbled through the growth medium was carefully measured. All cultures were inoculated with 10^7 conidia per l. except the 10 hr. culture which was started with 5×10^8 conidia per l.

Each sample of mold was incubated for 30 min. with 1 μc. of C^{14}-lysine (μl) (New England Nuclear) per g. wet weight. Before grinding, small samples of tissue were removed for dry weight determinations. An average of 85% of the sample weight was lost when dried in vacuum over $CaCl_2$. Cytochrome c was isolated as described (Methods). The cytochrome c content was estimated from difference spectra after Step II of the

TABLE 3

DISTRIBUTION OF C^{14}-LYSINE IN POKY

Fraction	Per cent of Label Taken Up
Small Metabolites	55.1
Lipids	0.01
Nucleic Acids	13.7
Proteins	32.4

A 127 mg. sample of poky mycelium was pulsed for 15 min. with 0.05 μc of C^{14}-lysine. The sample was extracted according to the procedure of Roberts et al. (74) to separate the various classes of molecules The distribution of C^{14}-lysine among the resulting fractions is reported as the percent of the C^{14} that was taken up by the cell during the pulse.

purification procedure. Both the supernatant and precipitate were analyzed.

Since conidia were disrupted with a low efficiency, the zero time determination of cytochrome c is, at best, only an estimate. As shown in Fig. 5 it is evident that the cytochrome c content of poky exhibits an initial, rapid rise during conidial germination (at 10 hrs. 17% of the conidia had germinated). By 22 hrs. or early logarithmic growth the poky cytochrome c complement reaches a maximum of 3% of the dry weight, then declines. This is in agreement with previous findings which demonstrated that the poky phenotype approaches a wild type state as the mutant ages (12).

From the total cytochrome c content and the ratio of C_I and C_{II} determined from chromatographic elution profiles, a time course for the C_I and C_{II} content was calculated (Fig. 5). A sequential synthesis of the two proteins is evident. In young poky during the period of cytochrome c accumulation (from 10 to 25 hrs.) only C_{II} is present. Significant amounts of C_I were first found in logarithmic cultures (38 hrs.). After this time the cellular concentration of C_{II} decreases as C_I becomes the dominant protein. Most important is the fact that the appearance of C_I coincides with the decrease in amounts of C_{II}. This observation led to the hypothesis that the synthesis of C_I and of C_{II} are related.

The specific activity of cytochrome c follows the same general pattern (Fig. 5). The initial value at 10 hrs. is high; however, values then decline and become essentially constant. The decline of the specific activity of C_I and C_{II} is equal. The pattern of amino acid incorporation as a function of time argues that the decline of cytochrome c content of poky is the result of decreased protein synthesis and not degradation.

Figure 5. Amounts of total cytochrome c, C_I and C_{II}; and the rate of cytochrome c synthesis at different ages of poky. Total cytochrome c was estimated in poky extracts after Step II of the purification procedure by difference spectra (Methods). The ratio of C_{II} and C_I was determined from chromatographic elution profiles. Amounts of C_I and C_{II} were calculated from the total cytochrome c content and the ratio of C_I and C_{II}. Cytochrome c is represented by circles.

Each sample of poky was pulse-labelled for 30 min. with 1 μc. of C^{14}-lysine per g. of tissue (wet weight). The specific activity of the cytochrome c was determined after Amberlite CG-50 chromatography. Specific activities are represented by triangles.
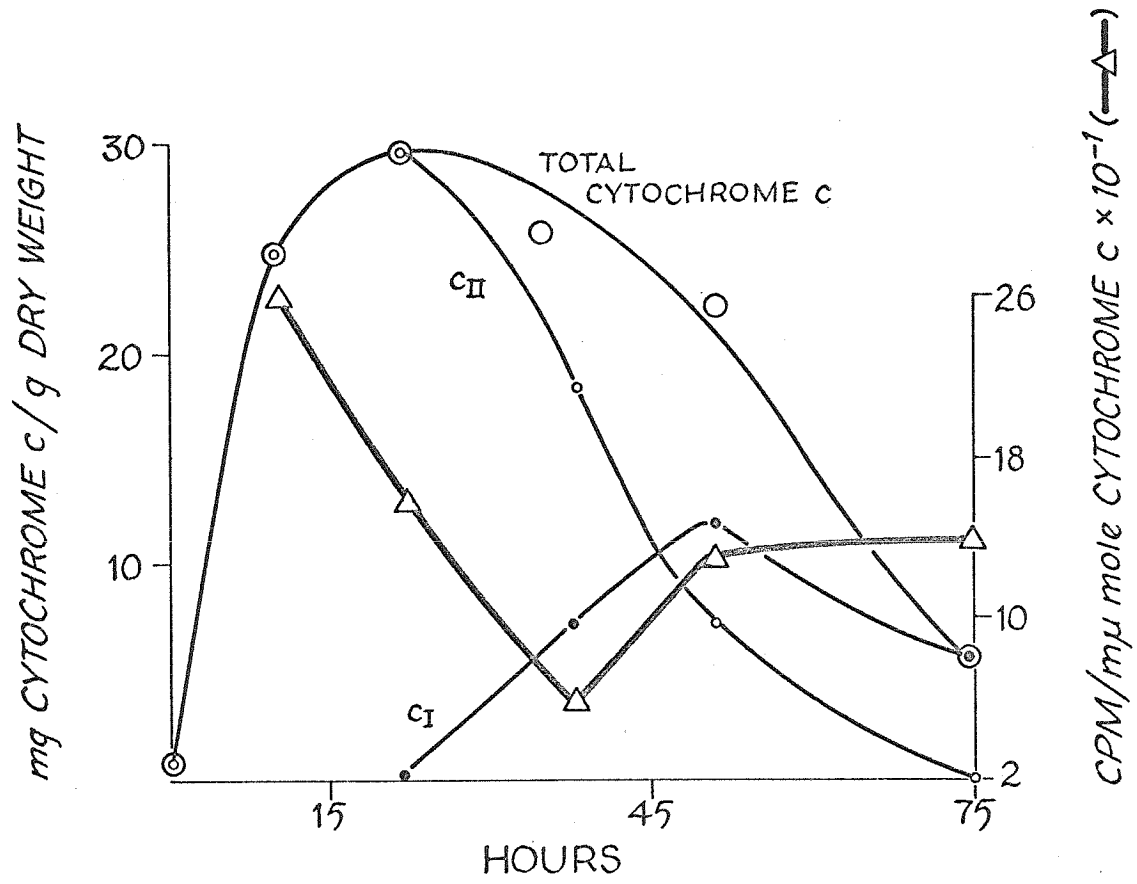
C_I and C_{II} in other Respiration Deficient Mutants and Wild Type

Cytochrome c was extracted from pre-log, log and post-log cultures of wild type, mi-3, cyt-1 and pof for the purposes of comparison with the results described for poky. It was of interest to determine if two cytochromes c are the result of the poky mutation and, if not, if C_I and C_{II} are synthesized in a sequential fashion as in poky. The cytochrome c content of the various strains was determined as described for poky and are given in Table 4. As previously observed mi-3 and cyt-1 have a higher cytochrome c content than wild type (13). It is not known, however, whether the variation in values obtained for mi-3 and cyt-1 represent a fluctuation in cytochrome c accumulation as found for poky. From the values in Table 4 the cytochrome c content for mi-3, cyt-1 and poky was calculated to be 2.4, 3.8 and 13 times higher than that of wild type.

C_I and C_{II} were detected in all ages of these strains, thereby proving that two cytochromes c are not the result of a poky mutation. In contrast to poky, however, C_{II} is a minor component of the cytochrome c complement of these strains even in pre-log cultures. The ratio of C_{II} and C_I is plotted as a function of age and is compared with values for poky in Fig. 6. Although mi-3 and cyt-1 accumulate cytochrome c, the values of C_{II}/C_I are identical to wild type. The values for suppressed poky are intermediate. Thus, the poky mutation appears to have two effects on cytochrome c synthesis: a) an accumulation of cytochrome c, and b) a delay of C_I synthesis. The other respiration deficient strains, mi-3 and cyt-1 , accumulate lesser amounts of cytochrome c but exhibit normal kinetics of C_I and C_{II} synthesis.

TABLE 4

CYTOCHROME c CONTENT OF VARIOUS NEUROSPORA STRAINS

Strain	Cytochrome c (Per Cent Dry weight)
Wild Type	0.25
mi-3	0.44 to 0.61
C-115	0.78 to 0.94
po-f	0.4 to 0.6
poky	0.51 to 3.0

Cytochrome c was estimated as per cent dry weight of tissue by difference spectra (71) in supernatant and precipitate derived from Step 2 of the purification procedure.

Figure 6. Ratio of C_I and C_{II} in various ages of wild type and several respiration deficient mutants of _Neurospora_. Cytochrome c was isolated from each sample of mycelium by base extraction then chromatographed on Amberlite CG-50 (Methods). The ratios of C_{II} and C_I were determined from chromatographic elution profiles. The ratios are plotted in terms of the phase of growth.
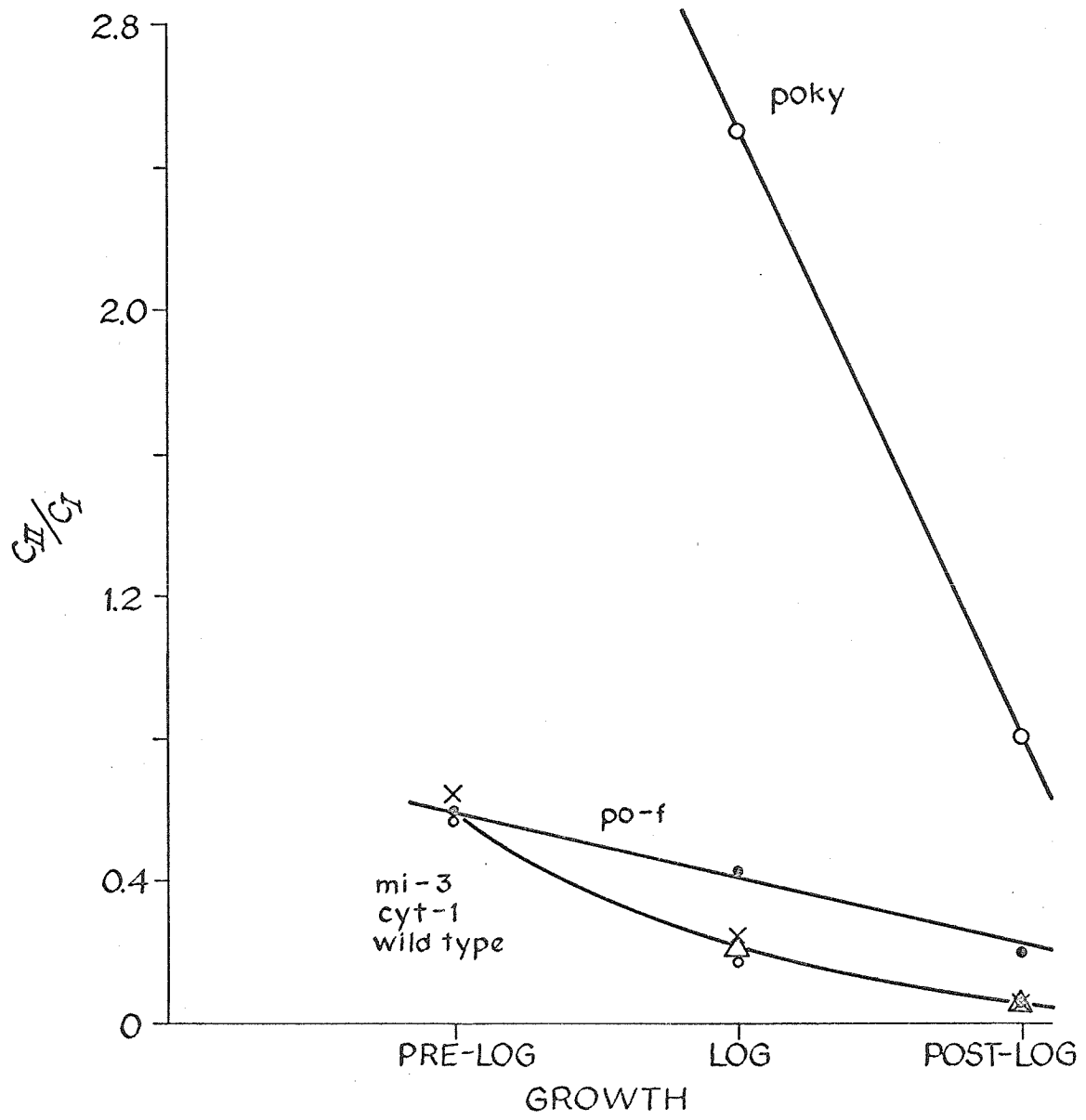
Conversion of C_{II} to C_I

A detailed study was undertaken to deduce the relationship between C_I and C_{II} synthesis. A temporal separation of C_I and C_{II} synthesis occurs in <u>poky</u> and, in addition, the decline of C_{II} content coincides with the appearance of C_I (Fig. 5). From these observations two possible relationships were considered: a) a precursor-product relationship between C_I and C_{II} or, b) <u>de novo</u> synthesis of both proteins with C_{II} acting as an inhibitor of C_I synthesis as postulated for the iso-cytochromes of yeast (54). To distinguish between these two models, three different kinds of experiments were performed: a) effect of acti-dione on the change of C_{II} to C_I, b) pulse-chase and c) kinetics of C^{14}-lysine incorporation into both proteins. The results of all three experiments are in accord with a precursor-product relationship between C_I and C_{II}.

<u>Conversion in the Presence of Acti-Dione:Controls</u> Acti-dione ranging in concentration from 0 to 1 µg./ml. was added to a series of <u>poky</u> cultures in 125 ml. flasks. Each culture was pulsed with an equivalent amount of C^{14}-lysine for 15 min. Protein was extracted by the procedure of Roberts <u>et al.</u> (74). The results are plotted in Fig. 7 as per cent incorporation of the control (no acti-dione) versus acti-dione concentration. As shown, the slope of the curve decreases rapidly to 40% inhibition at 0.2 µg./ml. of acti-dione then more slowly until protein synthesis is 90% inhibited at 1 µg./ml. of the drug.

The effect of various concentrations of acti-dione on the growth of <u>poky</u> was also tested. The same concentration range of acti-dione was employed as above with <u>poky</u> cultures grown in 125 ml. flasks. The results are shown in Fig. 8. Each determination represents the average weight

Figure 7. Acti-dione inhibition of protein synthesis and growth of poky.
Equal weight samples of poky were suspended in 20 ml. of Vogel's medium
N plus 2% sucrose then preincubated for 10 min. with the appropriate
concentration of acti-dione. Each was pulse-labelled for 10 min. with
1 μc of C^{14}-lysine. After harvesting on a Buchner funnel and washing
with distilled water, the tissue was suspended in cold TCA ($0°C$). Pro-
tein was extracted by the procedure of Roberts et al. (74). Radio-
activity was determined in the protein fraction extracted from each
sample after NaOH hydrolysis. The results are plotted (open circles)
as per cent of the radioactivity found in the protein fraction extracted
from the control (no acti-dione).

Also plotted for comparison is the inhibition of growth (solid
circles) as per cent of the control. These data were taken from the
65 hr. determinations shown in Fig. 8.
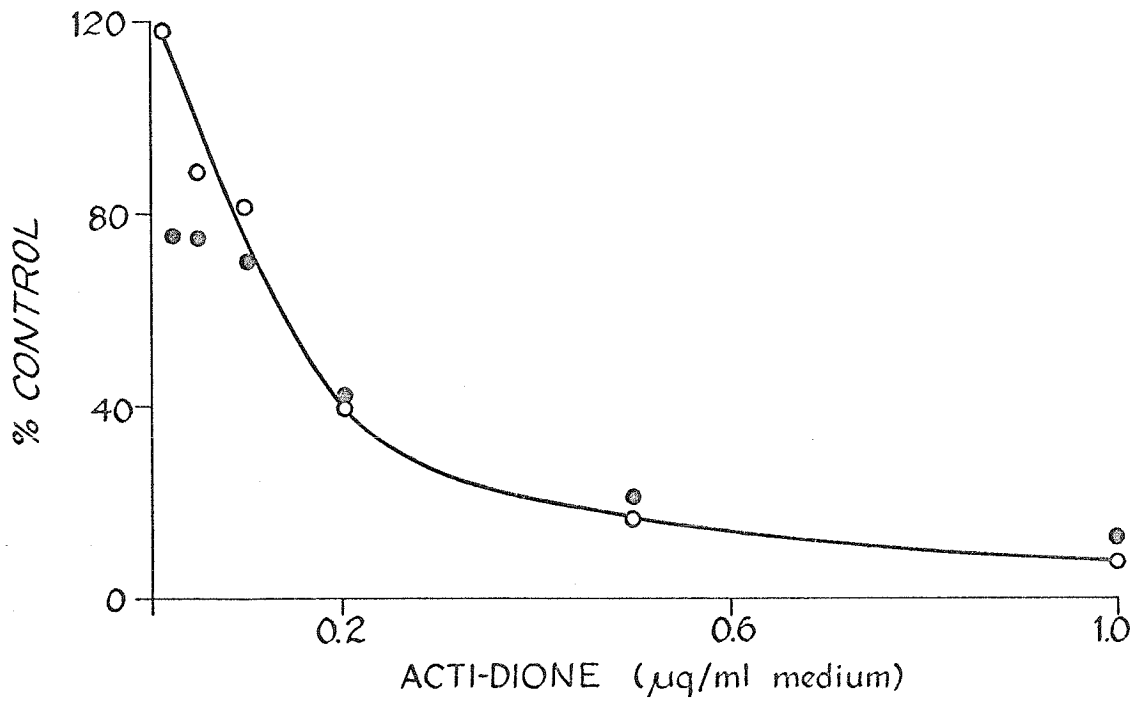
of mycelium from one flask. Acti-dione inhibits growth and protein synthesis to the same extent (see Fig. 7).

Effect of Acti-Dione on Cytochrome c Synthesis   A sample of 40 hr. poky was divided into three equal parts. Each sample was pulsed for 15 min. with equivalent amounts of C^{14}-lysine (1μc.) in the presence of 0.2, 0.1 and 0 μg./ml. acti-dione. Cytochrome c was purified (Methods) and the specific activity determined.

The incorporation of C^{14}-lysine into cytochrome c was found to be more sensitive to acti-dione than the average protein of poky. For example, at 0.2 μg./ml. of acti-dione no C^{14}-lysine was incorporated into cytochrome c although total protein synthesis was inhibited by only 60% (see Fig. 7). Similarly, cytochrome c synthesis was inhibited 97% by 0.1 μg./ml. of the drug (total protein synthesis was inhibited 25%).

Long Term Incubation of Poky with Acti-Dione: Effect on the Ratio of C_{II} and C_I and Total Cytochrome Synthesis   The rationale of these experiments was to incubate poky with acti-dione until, under normal circumstances (i.e. no acti-dione), a significant fraction of C_{II} is converted to C_I. Sufficiently high concentrations of acti-dione were employed to inhibit the synthesis of both proteins. Under these conditions the ratio of C_{II} and C_I should remain constant if the two proteins are the products of separate genes. Assuming, however, a precursor-product relationship between C_I and C_{II}, acti-dione may not inhibit the conversion of C_{II} to C_I provided the capacity for this reaction is present. It was assumed that if both proteins are present, the capacity to convert C_{II} to C_I is also present.

In the first experiment a culture of 40 hr. poky was divided into

Figure 8. Inhibition of poky growth by acti-dione. The indicated con-
centrations of acti-dione were added to 125 ml. flasks containing 20 ml.
of Vogel's medium N plus 2% sucrose. Each flask was inoculated with
2×10^6 poky conidia. Flasks were harvested at the indicated times.
Each determination is the dry weight of mold from one flask.
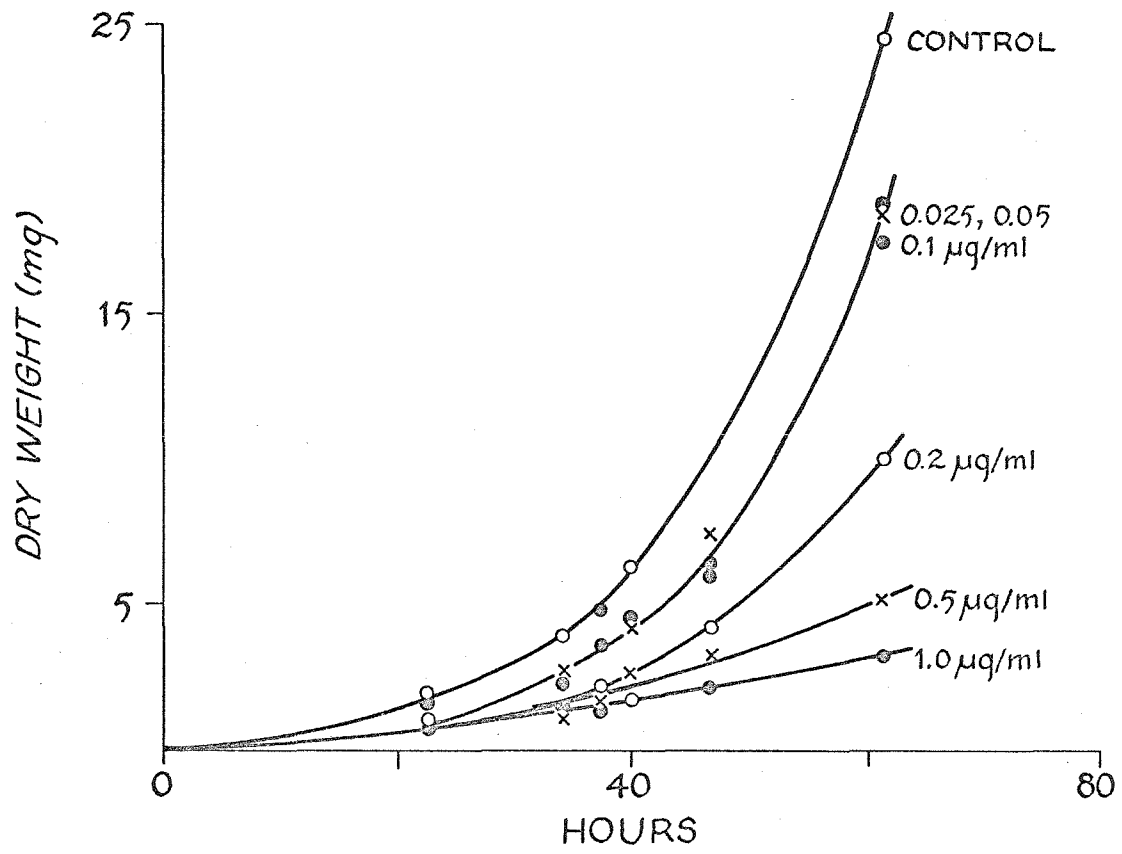
three equal parts; one part was harvested immediately (0 hr. control), the second was incubated for 12 hrs. in Vogel's medium N with 1 μg./ml. of acti-dione (acti-dione sample) and the third sample was grown for 12 hrs. in Vogel's medium N without acti-dione (12 hr. control). The total cytochrome c content was determined for each sample as described (p. 46). After cytochrome c isolation, the ratio of C_{II} to C_I was determined from chromatographic elution profiles.

Compared to the zero hr. control, the 12 hr. control increased in weight by a factor of 3 and in cytochrome c content by 2.9. In the same 12 hr. interval the ratio of C_{II} and C_I decreased by a factor of 2.1. On the other hand, by the same criterion the acti-dione sample did not increase in weight or cytochrome c content although the ratio of C_{II} and C_I decreased by a factor of 2.2.

The fact that the cytochrome c content and the weight of the acti-dione sample did not increase was taken as an indication that protein synthesis was effectively inhibited. Even so, the ratio of C_{II} and C_I continued to decrease by the same amount (a factor of 2) as did the ratio in the 12 hr. control in which cytochrome c synthesis was not inhibited.

The same experiment was repeated and enlarged. The 40 hr. poky culture was divided into four equal parts instead of three. The extra sample was incubated with 10 μg./ml. of acti-dione. In addition C^{14}-lysine was added to all samples during the 12 hr. incubation.

Again the results were the same for the 1 μg./ml. acti-dione sample, 0 hr. control and 12 hr. control. In the presence of 10 μg./ml. of acti-dione there was no increase in weight during the 12 hr. incubation. However, the total amount of cytochrome c decreased by 50% probably due to degradation as a result of cell death. An attempt was made to purify

the cytochrome c from this sample, but it failed since the protein
would not adhere to Amberlite CG-50.

Incorporation of C^{14}-lysine into total protein was measured by
taking 100 mg. pieces of tissue from all samples and extracting protein
by the procedure of Roberts et al. (74). These data indicated that
protein synthesis in the 1 μg./ml. and 10 μg./ml. acti-dione samples was
inhibited by 40 and 75% compared to the 12 hr. control. From this ob-
servation, then, it would appear that protein synthesis is not completely
inhibited at these concentrations of acti-dione. This is not surprising
since large amounts of poky (20g.) were employed in each incubation, and
since the results in Fig. 8 indicate that poky has the ability to over-
come the effect of acti-dione over long periods of time. However, from
the results of the effect of acti-dione on cytochrome c synthesis (i.e.,
cytochrome c synthesis was inhibited 97% when protein synthesis was in-
hibited only 25%) and the observation that the total amount of cyto-
chrome c did not increase in the 1 μg./ml. acti-dione sample, it can
be concluded that the conversion of C_{II} to C_{I} takes place in the absence
of appreciable cytochrome c synthesis.

Pulse-Chase Experiments These experiments were designed to examine
the fate of C^{14}-C_{II} in vivo. To obtain radioactive C_{II}, young poky
(10 hrs.) was pulsed with C^{14}-lysine. The fate of the label was deter-
mined after the C^{14} was chased with a thousand fold excess of C^{12}-lysine,
and the mold was allowed to grow to an age when the cytochrome c comple-
ment is only C_{I}. Assuming, then, that C_{I} is synthesized de novo from
amino acids, any incorporation of C^{14} into C_{I} would result from turnover of
C_{II} and other proteins of the cell. But, if C_{II} is a precursor of C_{I},

C^{14} originally found in C_{II} would be transferred quantitatively to C_I. In this case the specific activities of the two proteins should be comparable after adjusting for the amount of cytochrome c synthesis during incubation.

In the first experiment 16 l. of Vogel's medium N was inoculated with 3.7 g. of <u>poky</u> conidia and allowed to grow for 10 hrs. During this time the weight of the conidia increased to 10 g. The 10 hr. sample of <u>poky</u> was pulsed for 30 min. with 12 µc. of C^{14}-lysine; the C^{14} was chased with a thousand fold excess of C^{12}-lysine for 30 min. After dividing the sample into equal parts, one half was added to each of two carboys containing 16 l. of Vogel's medium N each. Following 20 hrs. of growth the mycelium from both carboys was harvested. The yield was 208 g. wet weight of tissue.

Only C_{II} was detected in the cytochrome c isolated from 2 g. of the initial 10 g. sample of partially germinated conidia. During the subsequent 20 hrs. growth, however, the amount of cytochrome c increased by 5.7 fold and the cytochrome c complement was converted to C_I. In the same interval, the cytochrome c specific activity decreased from 18,000 to 3,500. On the basis of the cytochrome c increase (5.7 fold), the specific activity would be expected to decrease to 3,140 from dilution assuming, of course, that C_{II} is a precursor of C_I.

A second and larger experiment of the same type was carried out. After germination, conidia (13.3g.) were pulsed with 15 µc of C^{14}-lysine. The C^{14} was chased with a thousand-fold excess of C^{12}-lysine. Again the sample was divided into equal parts; one half was taken for cytochrome c isolation (0 hr. sample). The remaining part was divided between two

carboys (3.5 g. to each) containing 16 l. of medium each. One carboy was harvested after 10 hrs. growth (16.1 g.) (10 hr. sample) and the second after 20 hrs. (82.5 g.) (20 hr. sample). Cytochrome c was extracted from each of the three samples.

From 0 to 10 hrs. growth after the pulse-chase, the total amount of cytochrome c increased by a factor of 5.5 but the specific activity decreased by only 2.2 fold. Both the 0 and 10 hrs. samples contained only C_{II}. During subsequent growth from 10 to 20 hrs. the amount of cytochrome c increased by another factor of 3.4 and the specific activity decreased by a comparable amount (3.5 fold). All of the cytochrome c was converted from C_{II} to C_I during this latter interval.

A small piece of tissue was removed from each of the three samples and extracted by the procedure of Roberts et al. (74). From these data it was ascertained that the chase with C^{12}-lysine was ineffective. The free amino acid pool of the 0 hr. sample contained 50% of the radioactivity taken up by the cell (compare with Table 3). In the 10 hr. sample the radioactivity of the free amino acid pool had decreased to 5% of the total C^{14}. The discrepancy between the increase in cytochrome c content and decrease in specific activity from 0 to 10 hrs. growth, then, can be explained by an incorporation of C^{14}-lysine from the free amino acid pool. More important, however, the specific activity and the total number of counts in cytochrome c remained constant during the time (10 to 20 hrs.) the cytochrome c complement of poky changed from C_{II} to C_I. This result and the result from the previous pulse-chase experiment are compatible with the idea that C_{II} is a precursor of C_I.

The same experiment was attempted in a slightly different way but

without success. Poky conidia were incubated in Vogel's medium N with D, L-lysine, 4, 5-H^3 for 10 hrs. After harvesting and washing the spores, the H^3 was chased with a 10^5 excess of H-lysine. The sample was then added to 16 l. of medium. One half was harvested after 24 hrs. of growth and the remainder after 36 hrs. At 24 hrs. all of the cytochrome c was C_{II} but only C_I was found at 36 hrs. During this interval the weight and total amount of cytochrome c increased by 5 fold, although the specific activity decreased by only 2.5 fold. The discrepancy between the cytochrome c increase and specific activity decrease is probably the result of an ineffective chase as shown for the 0 to 10 hrs. samples in the previous experiment. The discrepancy, it should be noted, is that a greater amount of label was found in C_I than predicted. The result still argues for the precursor-product relationship for C_I and C_{II}. It is difficult to imagine how the total amount of cytochrome c can increase by 5 fold while the specific activity decreases by only 2.5 times that of the original cytochrome c, unless the amount of label in the original cytochrome c is retained.

Kinetics of Amino Acid Incorporation into C_I and C_{II} In Vivo The precursor-product relationship between C_I and C_{II} was also shown in the following manner. The rate of C^{14}-lysine incorporation into the two proteins was compared in 55 hr. poky (C_I and C_{II} are presented in approximately a ratio of 2 to 1). Poky (40 g.) was pulsed for varying periods of time ranging from 0 to 60 min. with 50 μc of C^{14}-lysine. Samples were removed at the specified time intervals (Fig. 9); the cytochrome c was purified and the specific activity of both proteins determined. From the results presented in Fig. 9 the flow of radioactivity from C_{II} to C_I is evident. The incorporation of C^{14}-lysine into both proteins is

Figure 9. Kinetics of C^{14}-lysine incorporation into C_I and C_{II}.

To 40 g. of 55 hr. poky was added 50 μc. of C^{14}-lysine (u.l.). Samples were removed at the appropriate time intervals and the cytochrome c was purified (Methods). The amount of radioactivity in C_I and C_{II} was determined from aliquots of the appropriate fractions from the Amberlite CG-50 column. The reported values are an average specific activity of the C_I and C_{II} fractions from each column.
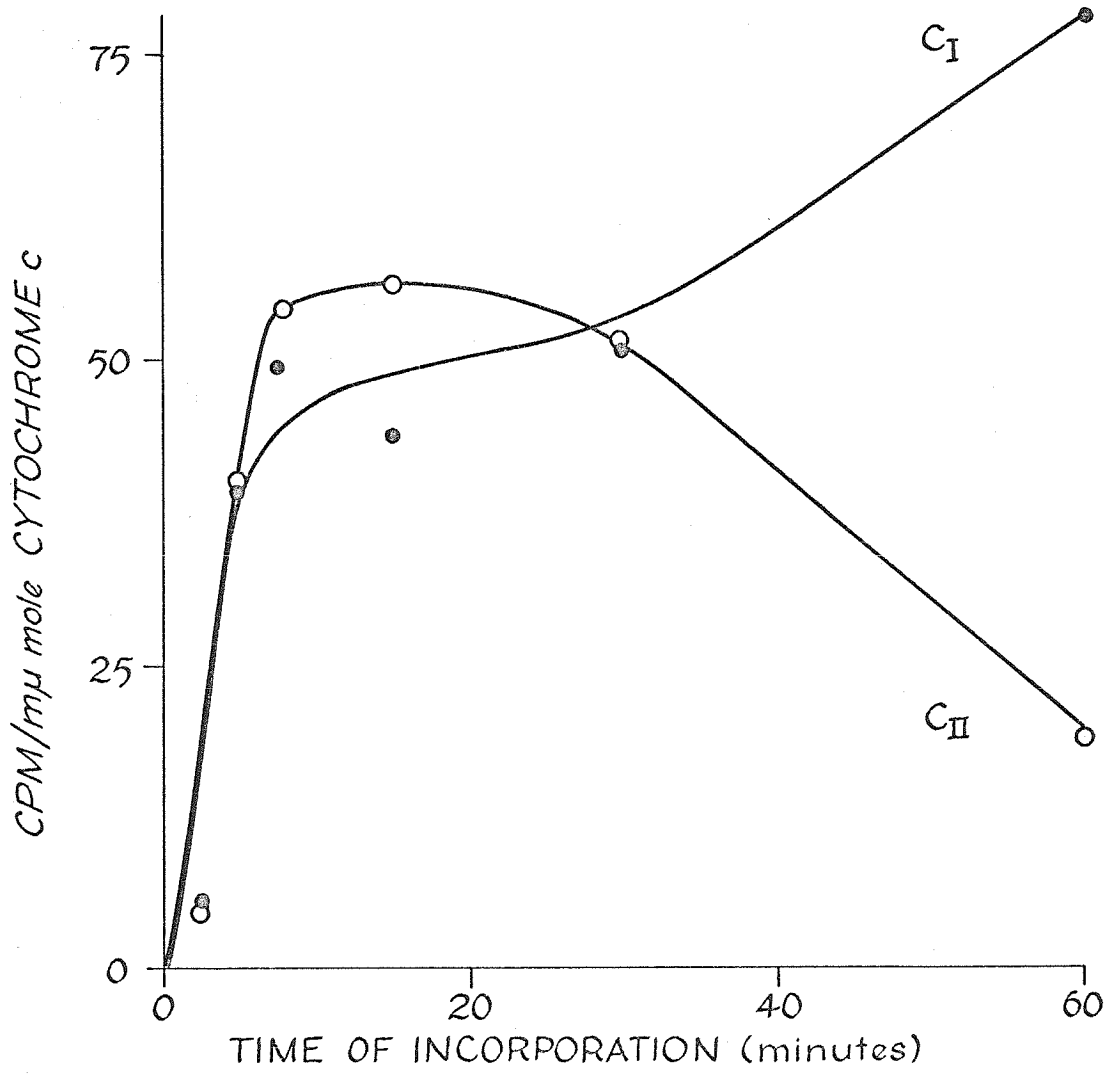
comparable during the first 30 min. of labeling. Beyond this time the C^{14}-lysine of the free amino acid pool is presumably depleted since the specific activity of C_{II} decreases. In contrast, the specific activity of C_I continues to increase from 30 to 60 min. Significant to this argument is the fact that the decrease of radioactivity in C_{II} is comparable to the increased amounts found in C_I.

Structural Difference Between C_I and C_{II}

A detailed study of peptides derived from C_I and C_{II} was undertaken and is described here. In agreement with the above findings this study confirmed that C_I and C_{II} are different in primary structure. The difference, however, is a secondary modification. C_{II} contains lysine at residue 72 but C_I has a basic amino acid whose electrophoretic mobility is different from any common amino acid. The odd amino acid in C_I is shown to be a lysine derivative, thus confirming the precursor-product relationship between C_I and C_{II}.

Fortunately, during the early part of this study Heller and Smith (59) published the complete amino acid sequence of C_I (see Fig. 10). (For proof that C_I and the cytochrome c sequenced by Heller and Smith (59) are the same see p.38). Tryptic peptide maps, tryptic, cyanogen bromide and chymotryptic peptides of C_I and C_{II} were compared. Initially only the C_{II} peptides were extensively analyzed and compared to those published by Heller and Smith for C_I. The finding of the modified lysine residue in C_I made it necessary to analyze tryptic peptides from C_I also.

Since C_{II} is the minor component of the two cytochromes c in all but the earliest stages of poky growth, techniques were scaled down to accommo-

Fig. 10. The complete amino acid sequence of Neurospora crassa cyto-
chrome c as determined by Heller and Smith (59). The tryptic peptides
(T————) are shown above the sequence and the chymotryptic peptides
(C ---------) and cyanogen bromide peptides (CNBr ———) are shown below.
The cleavages are those found in the present studies. The numbering
of the residues is described by Margoliash and Schejter (1).

```
|————————————————T-1————————————————|  |———————————T-2———————————|—————T-3—————|

NH₂ Gly  Phe Ser Ala  Gly Asp Ser Lys Lys  Gly Ala Asn Leu Phe Lys Thr Arg
      -4            1                                      10
|——C-1————————|————————————————————————C-2—————————————————————————|
```

```
|——————————————————————————————T-4————————————————————————————|

Cys Ala Gly Cys His Gly Glu Gly Gly Asn Leu Thr Gln Lys Ile Gly Pro Ala
    HEME              20                                          30
                                      |————————————————C-4—————————
```

```
|———T-5———————————————|  |——————————————————————T-6————————————————|

Leu His Gly Leu Phe Gly Arg Lys Thr Gly Ser Val Asp Gly Tyr Ala Tyr Thr
                            40
————————|————C-5————|—————————————————————C-6—————————————————|——C-7——|————
```

```
                |——T-7——|

Asp Ala Asn Lys Gln Lys Gly Ile Thr Trp Asp Glu Asn Thr Leu Phe Glu Tyr
50                               60
————C-8————————|————————C-9—————————|————————C-10—————————|————————
                                                          |——C-11a'-
```

```
        | |——————————T-9—————————|——————————T-10—————————

Leu Glu Asn Pro Lys Lys Tyr Ile Pro Gly Thr Lys Met Ala Phe Gly Gly Leu
        70                                  80
—————|———C-11—————————————|—————————C-12————————|——C-13—|———C-14————|
—————|————————————C-11c——————————|
```

```
—| |——T-11—|                          |——————————T-14————————|

Lys Lys Asp Lys Asp Arg Asn Asp Ile Ile Thr Phe Met Lys Glu Ala Thr Ala COOH
          90                              100         103
—————————————————————C-15—————————————————|——|————————C-16——————————|
——————————————————CNBr-2——————————————————|——|————————CNBr-3————————|
```

date small quantities of protein. Over 200 l. of early log phase poky

were grown to isolate 50 mg. of C_{II}; this study was carried out on this

amount of protein.

Peptide Maps   Peptide maps of tryptic digests of C_I and C_{II} were

carried out as described under Methods. The tryptic digestions, chroma-

tography and electrophoresis were run in parallel. Photographs and tracings

are shown in Fig. 11. It is evident that the two peptide maps are essen-

tially identical. Twenty-two of the twenty-three ninhydrin positive spots

match. A single spot marked by cross-hatching in the tracing of the C_I

peptide map is missing from the C_{II} map.

Total Amino Acid Composition of C_{II} The amino acid composition of

C_{II} as determined by column analysis is shown in Table 5 and is compared to

the composition determined for C_I by Heller and Smith (59). The compo-

sition of C_{II} is in good agreement with that of C_I. It was assumed that a

single tryptophan residue is present on the basis of a single Ehrlich

positive chymotryptic peptide.

Tryptic Peptides   The elution patterns of C_I (30 mg.) and C_{II}

(25 mg.) tryptic digests from Dowex-50 are shown in Fig. 12.

The nomenclature for peptides is similar to that used by Heller and

Smith (59). In general a tryptic peptide is designated by T-. Each pep-

tide is given a number according to its position from the N-terminal end

(T-1). In the numbering system used by Heller and Smith (59) free lysine

residues were included; in the present system they are not. In descriptions

of peptide purification no distinction is made between C_I and C_{II} since

a specific peptide from either protein generally followed the same

pattern. In cases where distinction is made between equivalent peptides

Figure 11. Peptide maps of trypsinized C_I and C_{II}. On the left are photographs of the maps stained with the collidine-ninhydrin reagent (Table 1). On the right are tracings of these photographs.
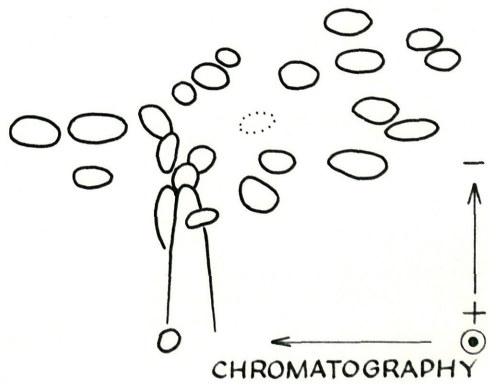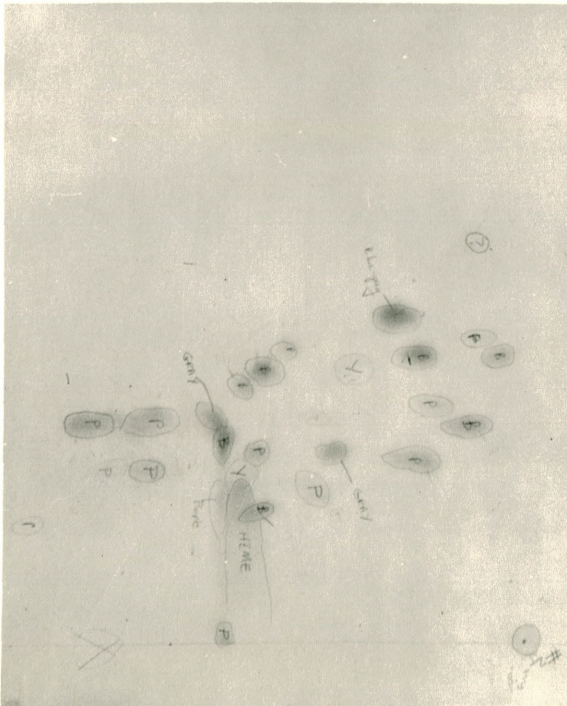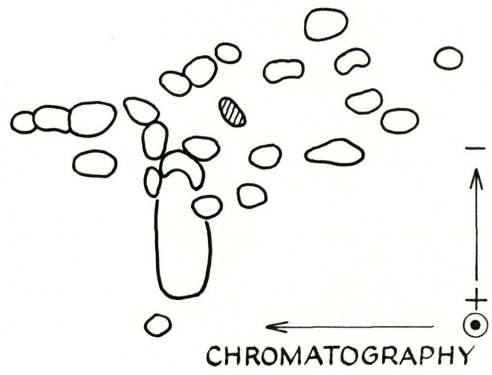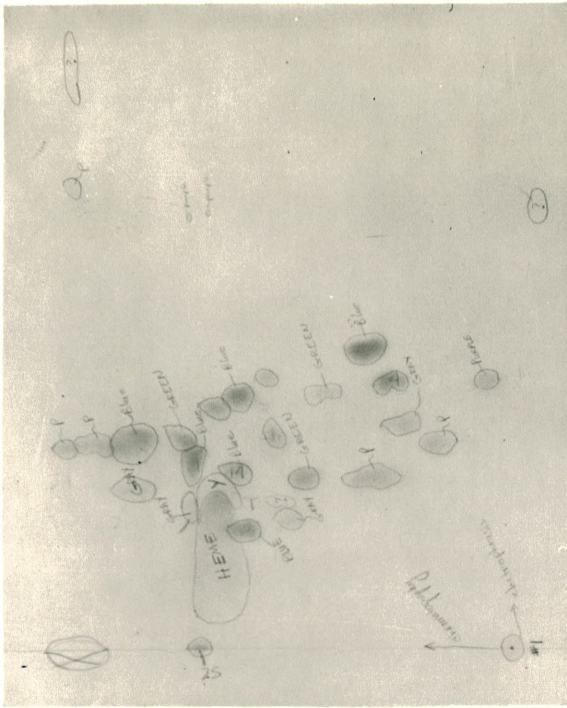
TABLE 5

AMINO ACID COMPOSITION OF C_I AND C_{II}

Amino Acid	C_{II}	C_I [1]
Lysine	13.0	14.4
Histidine	2.0	2.1
Arginine	2.8	3.2
Aspartic acid	13.7	13.5
Threonine	8.4	8.9
Serine	2.8	2.9
Glutamic acid	8.5	8.1
Proline	2.7	3.0
Glycine	15.2	15.3
Alanine	9.1	9.2
Half-Cystine	1.6	1.9
Valine	1.0	1.0
Methionine	1.6	1.9
Isoleucine	5.0	4.9
Leucine	6.8	7.1
Tyrosine	3.1	3.9
Phenylalanine	5.7	6.1
Tryptophan	1	1

[1] Values taken from Heller and Smith (59).

of the two proteins, the peptide from C_I is designated as T_1-1 and the peptide from C_{II} as T_{II}-1.

Only peptide T-14 required no further purification. Peptides T-12 and T-13 were recovered in insufficient quantities for amino acid analysis on paper. Peptide T-8 was detected in column pool 5 with Ehrlich's reagent, but the quantities were too small for amino acid analysis.

Most tryptic peptides were purified by two different methods. In these cases the compositions of the peptide isolated by each method were averaged.

Peptide T-1 This neutral peptide was separated from trace amounts of peptide T-13 by preparative chromatography-electrophoresis and by electrophoresis at pH 6.0 and pH 3.5.

Peptide T-2 Separation of this basic peptide from peptides T-7 and T-9 was carried out by preparative chromatography-electrophoresis and by pH 6.0 electrophoresis.

Peptide T-3 This basic peptide was separated from peptides T-12, T-2 and T-15 by pH 3.5 electrophoresis. When purified by pH 6.0 electrophoresis this peptide was contaminated with slight amounts of peptide T-7.

Peptide T-4 This acidic heme peptide was separated from minor contaminants by pH 3.5 electrophoresis. In contrast to the results of Heller and Smith (59) this peptide was eluted from the peptide column.

Peptide T-5 This basic peptide purified from small amounts of peptide T-4 by pH 6.0 and pH 3.5 electrophoresis corresponds to peptide T-6 of Heller and Smith (59) with the addition of Gly-Arg on the C-terminal

Figure 12. Elution patterns of peptides from tryptic hydrolyses of C_I and C_{II}. The digest was chromatographed on a Dowex 50-X2 column with pyridine-acetate buffers as described in METHODS. The solid lines indicate fractions pooled. The numbers refer to the peptides found in each fraction. See the text for nomenclature.

end. In addition to peptide T-5, a peptide with the composition of (Gly, Arg) was recovered in high yield. The latter peptide is derived presumably from peptide T-5 by a chymotryptic split at residue (Phe) 67. The other fragment of this split corresponding to peptide T-6 of Heller and Smith (59) was not detected.

Peptide T-6 This neutral peptide was separated from peptide T-15 and trace amounts of peptide T-13 and T-7 by pH 6.0 and pH 3.5 electrophoresis.
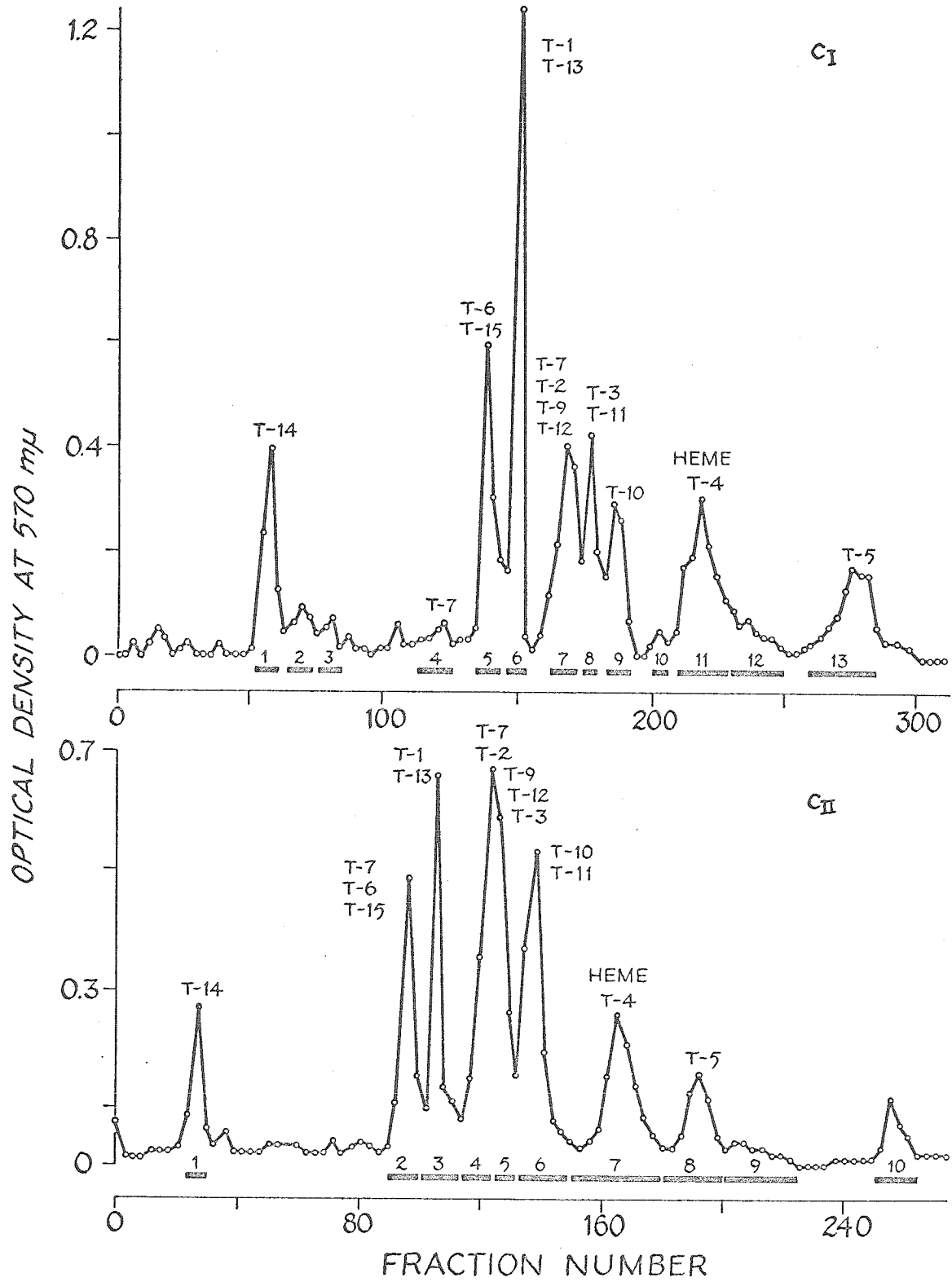
Peptide T-7 Separation of this neutral peptide from peptides T-2 and T-9 was carried out by preparative chromatography-electrophoresis and by pH 3.5 electrophoresis.

Peptide T-8 This neutral peptide from column pool 4 was separated from peptide T-2 by preparative chromatography-electrophoresis and from column pool 5 by pH 6.0 electrophoresis.

Peptides T-10 and T-11 These two neutral peptides were separated from each other on preparative chromatography-electrophoresis and by pH 6.0 electrophoresis.

Peptide T-15 Free lysine was separated from peptides T-12, T-13 and T-6 by pH 3.5 electrophoresis.

One major difference was found in the tryptic peptides isolated from C_I and C_{II} in this study and those isolated by Heller and Smith from C_I (59). A peptide having the sequence Asp-Lys-Asp-Arg corresponding to residues 88 through 90 was reported by these authors (see Fig. 10). In the present study the lysine bond at residue 89 was found to be sensitive to trypsin. The N-terminal half of this peptide was recovered (peptide T-11) although the C-terminal portion, Asp-Lys, (peptide T-12) was not detected.

The amino acid compositions of the C_{II} tryptic peptides are listed in Table 6. Amino acid analyses of peptides T-4 and T-6 were determined by column chromatography. The cysteine content of peptide T-4 was estimated by paper electrophoresis after converting cysteine to cysteic acid by performic acid treatment (83). All other peptides were analyzed by paper electrophoresis.

The amino acid compositions of C_{II} tryptic peptides are in good agreement with those published for C_I (59).

Cyanogen Bromide Peptides In order to isolate the peptide from the region of C_{II} (residues 90-98) corresponding to the missing tryptic peptides, T-12 and T-13, advantage was taken of the position of the two methionines (residues 90 and 98) in the protein. Under acid conditions cyanogen bromide is known cleave proteins at methionine carboxyl groups (91). The reaction of C_I and C_{II} with cyanogen bromide should yield three peptides: CNBr-3 (residues -1 to 80). CNBr-2 (residues 81 to 98) and CNBr-3 (residues 99-103) (59). Peptide CNBr-2 includes the residues of the missing tryptic peptides, T-12 and T-13.

Amino acid analyses of peptide CNBr-2 from C_I and C_{II} are given in Table 7. The compositions are identical. C_I and C_{II}, therefore, do not differ by an amino acid substitution in the segment of the polypeptide chain extending from residues 81 through 98.

Chymotryptic Peptides Since peptide T-8 from the tryptic digest of C_{II} was not recovered, the segment of the C_{II} amino acid sequence from residues 56 to 72 was not analyzed. From the data of Heller and Smith (59) chymotrypsin cleaves three bonds in this region producing three relatively short chymotryptic peptides. C_I (20 mg.) and C_{II} (22 mg.)

TABLE 6 AMINO ACID COMPOSITION OF C_{II} TRYPTIC PEPTIDES

AMINO ACID	T-1	T-2	T-3	T-4	T-5	T-6	T-7	T-8
LYSINE	1.0(1)	1.0(1)		1.0(1)		1.0(1)	1.3(1)	
ARGININE			1.1(1)					
HISTIDINE					1.0(1)			
GLYCINE	1.6(2)	1.0(1)		0.8(1)	1.0(1)	1.8(2)		
ALANINE	1.1(1)	0.9(1)		3.2(3)	2.7(3)	1.9(2)		
VALINE				1.0(1)	1.0(1)	1.3(1)		
SERINE	1.7(2)					1.0(1)		
ISOLEUCINE					1.0(1)			
LEUCINE		1.0(1)		1.2(1)	1.9(2)			
THREONINE			1.0(1)	0.8(1)		1.9(2)		
PROLINE								
METHIONINE								
GLUTAMIC ACID				3.1(1)				
PHENYLALANINE	1.1(1)	1.1(1)			1.0(1)		1.0(1)	
ASPARTIC ACID	1.2(1)	1.2(1)		1.1(1)		3.0(3)		
TYROSINE						1.5(2)		
CYSTEINE				1.6 (2)				

TABLE 6   AMINO ACID COMPOSITION OF C_{II} TRYPTIC PEPTIDES (CON'T.)

AMINO ACID	T-9	T-10	T-11	T-12	T-13	T-14	T-15
LYSINE	1.0(1)	1.1(1)	1.0(1)				4.0(4)
ARGININE							
HISTIDINE							
GLYCINE	0.9(1.0)	2.1(2)					
ALANINE		1.1(1.0)					
VALINE						2.2(2)	
SERINE							
ISOLEUCINE	1.0(1)						
LEUCINE		1.2(1.0)					
THREONINE	1.0(1)					1.0(1)	
GLUTAMIC ACID							
PHENYLALANINE		1.1(1)					
ASPARTIC ACID			1.1(1)				
TYROSINE	0.8(1.0)						
CYSTEINE							

The composition of each peptide is given as molar ratios of the amino acids without correction for destruction during acid hydrolysis. Peptides T-4 and T-6 were analyzed by column chromatography. All other peptides were analyzed by paper electrophoresis. Cysteine in peptide T-4 was determined by paper electrophoresis after conversion to cysteic acid by performic acid treatment (85). The values in parentheses are the number of residues determined by Heller and Smith for C_I (59).

TABLE 7

AMINO ACID COMPOSITION OF PEPTIDE CNBr-2 FROM CYANOGEN

BROMIDE CLEAVAGE OF C_I AND C_{II}

Amino Acid	C_{II}	C_I	Smith[1]
		Molar Ratio	
Lysine	2.9	3.1	3
Arginine	0.9	1.0	1
Glycine	2.0	2.3	2
Alanine	1.1	1.3	1
Isoleucine	2.0	2.4	2
Leucine	1.0	1.2	1
Threonine	1.0	1.0	1
Phenylalanine	2.2	2.1	2
Aspartic acid	3.6	3.7	4

C_I (5.8 mg) and C_{II} (3.3 mg) were treated with an 80 fold molar excess of cyanogen bromide in 70% formic acid. The reaction products were separated on a Sephadex G-50 column, then purified further by paper chromatography. Amino acid analyses were carried out by paper electrophoresis on peptide CNBr-2 from both proteins. [1] These values were determined by Heller and Smith (61) for the same peptide obtained by cyanogen bromide treatment of C_I.

were therefore hydrolyzed with chymotrypsin to isolate these peptides. The elution profiles from Dowex-50 columns are shown in Fig. 13.

Nomenclature for chymotryptic peptides is the same as that used for tryptic peptides except the former are designated by C- rather than T-. In describing pooled fractions from peptide columns, C_I-8 refers to protein C_I pool 8 and similarly for C_{II} (C_{II}-8).

Chymotryptic peptides were better separated on Dowex-50 than tryptic peptides. As a result all chymotryptic peptides except C-7 and C-13 were found to be pure after pH 3.5 electrophoresis. Peptides C-7 and C-13 migrated together at pH 3.5 but were separated by preparative chromato-graphy-electrophoresis. Amino acid compositions were determined by paper electrophoresis or a combination of chromatography-electrophoresis for those peptides that contained tyrosine and aspartic acid.

The amino acid compositions of C_{II} chymotryptic peptides are given in Table 8. The values agree with those published for C_I (59) except for peptide C-11a' which has the composition (Glu, Tyr, Leu). The sequence of peptide C-11a described by Heller and Smith (59) is Glu-Tyr and corre-sponds to residue 66 and 67 (see Fig 10). Since residue 68 is Leu it is assumed that peptide C-11a' is the result of a chymotryptic cleavage at Leu rather than Tyr. This assumption is based on the fact this se-quence is unique in the protein.

Peptides C-9, C-10, C-11, C-11a' and C-11c span the sequence of the missing peptide T-8. The amino acid analyses of the peptides agree with the published amino acid sequence of C_I (59). These results, together with the data from tryptic and cyanogen bromide peptides, eliminate the possibility of an amino acid substitution as a difference between C_{II}

Figure 13. Elution patterns of peptides from chymotryptic hydrolyses of C_I and C_{II}. The digest was chromatographed on a Dowex 50-X2 column with pyridine-acetate buffers as described in METHODS. The solid lines indicate fractions pooled. The numbers refer to the peptides found in each fraction. See the text for nomenclature.
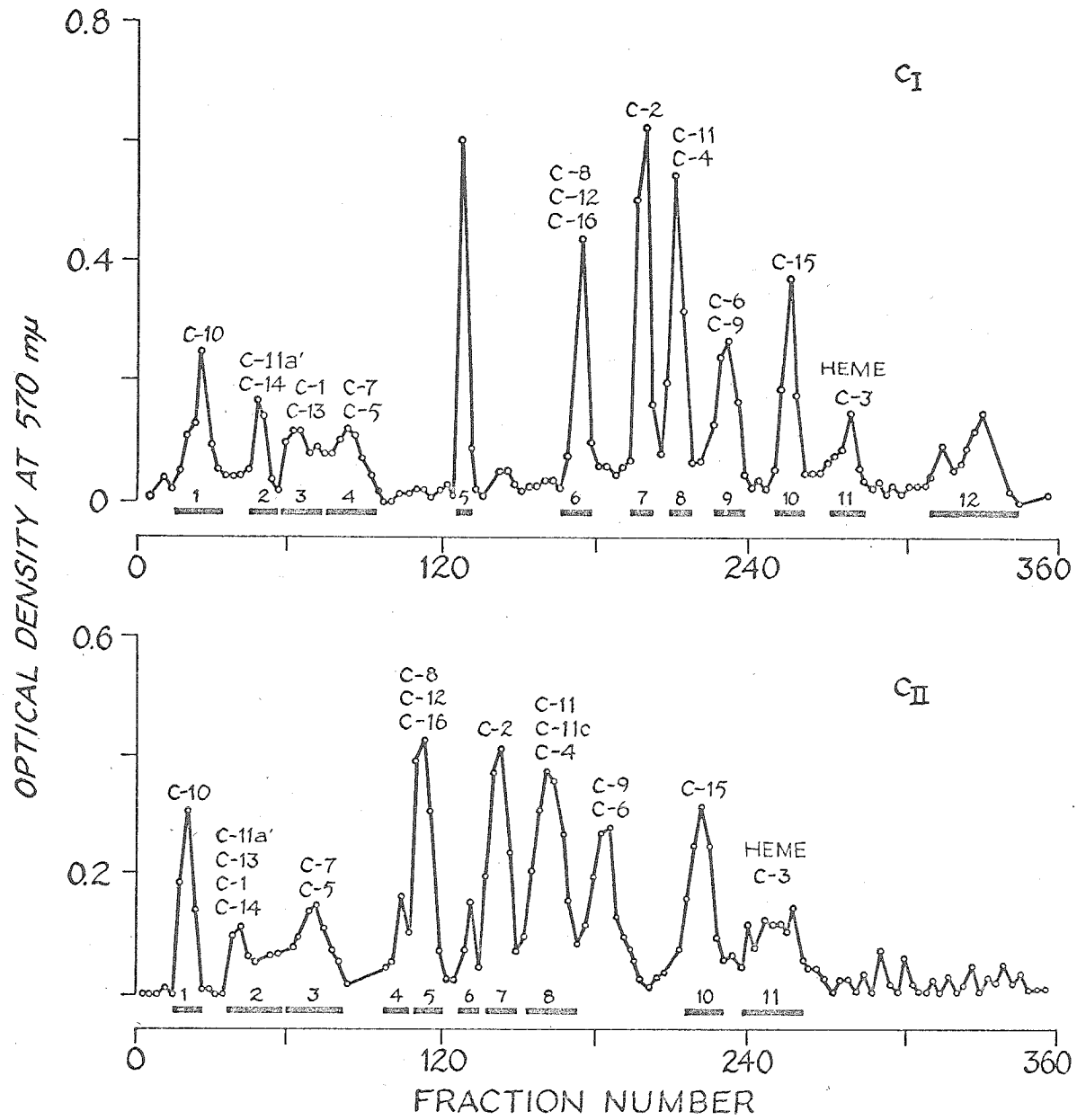
TABLE 8   AMINO ACID COMPOSITION OF C_{II} CHYMOTRYPTIC PEPTIDES

AMINO ACID	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9
LYSINE		1.7(2)				1.1(1)		1.0(1)	1.0(1)
ARGININE						1.0(1)			
HISTIDINE				1.4(1)					
GLYCINE	1.0(1)	1.9(2)		1.1(1)	1.0(1)	2.4(3)			0.7(1)
ALANINE		1.7(2)		0.9(1)			1.4(1)	1.2(1)	
VALINE		1.5(2)				1.3(1)			
SERINE						0.9(1)			
ISOLEUCINE				1.1(1)					1.4(1)
LEUCINE		1.0(1)		1.0(1)	1.0(1)	0.9(1)	1.2(1)		
THREONINE								0.9(1)	1.2(1)
PROLINE									
METHIONINE									
GLUTAMIC ACID									
PHENYLALANINE	1.0(1)	1.1(1)			1.2(1)	1.0(1)			
ASPARTIC ACID		1.7(2)				0.7(1)		2.0(2)	
TYROSINE							1.0(1)		

TABLE 8   AMINO ACID COMPOSITION OF C_{II} CHYMOTRYPTIC PEPTIDES (CON'T.)

AMINO ACID	C-10	C-11	C-11a	C-11c	C-12	C-13	C-14	C-15	C-16
LYSINE				2.0(2)	0.9(1)			2.6(3)	1.1(1)
ARGININE								1.0(1)	
HISTIDINE									
GLYCINE		1.7(2)							
ALANINE					0.9(1)		1.6(2)		2.0(2)
VALINE						1.0(1)			
SERINE									
ISOLEUCINE	1.1(1)				1.1(1)			1.5(2)	
LEUCINE	1.0(1)	1.0(1)	1.3(1)				1.0(1)		
THREONINE					1.2(1)				1.1(1)
PROLINE									
METHIONINE					1.2(1)				
GLUTAMIC ACID	1.1(1)	1.9(2)	1.0(1)	1.0(1)					1.1(1)
PHENYLALANINE	1.1(1)					1.3(1)		1.1(1)	1.0(1)
ASPARTIC ACID	1.7(2)	0.7(1)	0.8(1)	1.1(1)					
TYROSINE		1.6(2)		0.7(1)				3.8(4)	

The composition of each peptide is given as molar ratios of the amino acids without correction for destruction during acid hydrolysis. All peptides were analyzed by paper electrophoresis. The values in parentheses are the number of residues determined by Heller and Smith for C_I(59).

and C_I.

The above results, however, do not rule out a difference in amide content or an inversion of a short amino acid sequence in an overlapping region of tryptic and chymotryptic peptides. To eliminate these two cases, the electrophoretic mobilities of C_I and C_{II} chymotryptic peptides were compared at pH 1.9, pH 3.5 and pH 6.0. No mobility differences were found at pH 1.9 or pH 3.5. At pH 6.0 fraction C_I-1 had a ninhydrin positive spot with a mobility of a neutral peptide (Fig. 14). No significant quantitites of any amino acid were found after acid hydrolysis of the presumed peptide. It was therefore considered an artifact.

Fractions C_I-8 and C_{II}-8 are comparable column pools from the two proteins. At pH 6.0, fraction C_{II}-8 has an extra peptide compared to fraction C_I-8 (Fig. 14). Amino acid analysis of the peptides from the two fractions indicated that the extra peptide in fraction C_{II}-8 is peptide C-11c. This peptide was absent in the C_I chymotryptic digest. No other significant mobility differences were found.

From these data was concluded that C_I and C_{II} do not differ by an amide group since a difference in charge between corresponding peptides of the two proteins would have resulted in a mobility difference. The only case where the present data are not sufficient to establish a compatibility of sequence, is an inversion of a short amino acid sequence (48). Such an inversion is highly unlikely, however, since it would have to be such that the electrophoretic and chromatographic mobilities of the tryptic, cyanogen bromide and chymotryptic peptides as well as the amino acid composition of the peptides and the overall composition of the protein were not altered.

Figure 14. Electrophoresis at pH 6.0 of C_I chymotryptic peptides obtained

from column chromatography. The nomenclature refers to the column pools.

For example, C_{II}-1 refers to the pooled fractions of the first ninhydrin

positive peak eluted from the C_{II} peptide column. C_I-1 is the comparable

pooled fraction from the C_I peptide column. Electrophoresis was carried

out for 8 hrs. at 26.7 volts/cm. at 22-25°C. The cathode and anode are

designated in the figure. The peptides were detected by dipping the

paper in the collidine-ninhydrin reagent (Table 1).

G_I-1 G_I-1 G_{II}-2 G_I-2 G_{II}-3 G_I-3 G_I-4 G_I-4 G_I-5 G_I-6 C_{II}-7 G_I-7 G_I-8 G_I-8 C_I-9 C_I-9 G_{II}-10 C_I-10 C_{II}-11 C_I-11 C_{II}-12
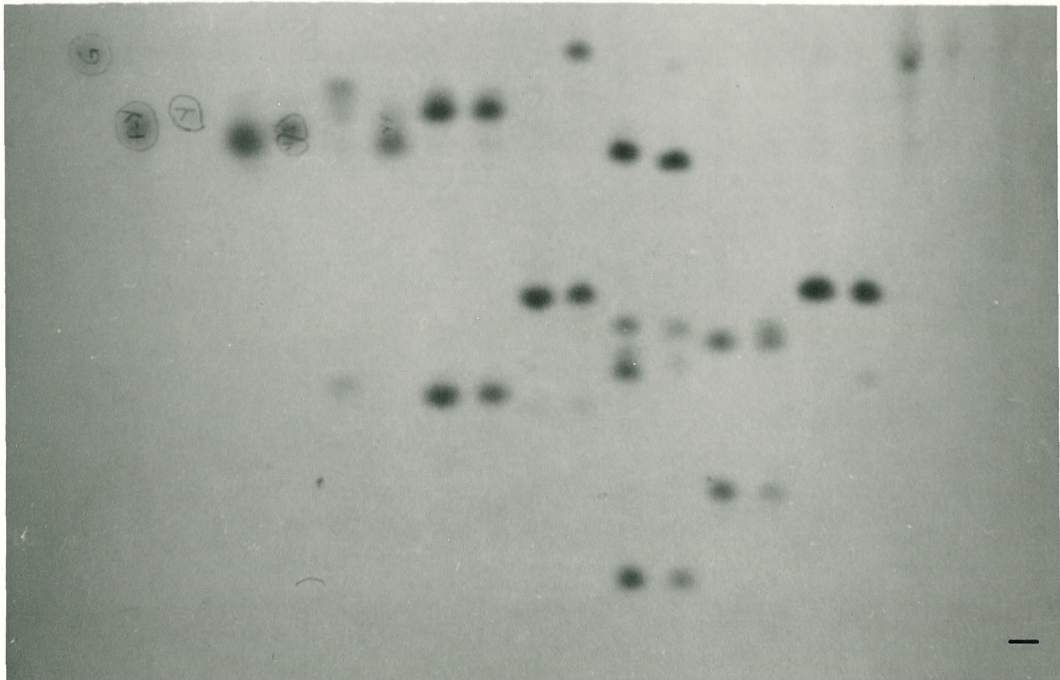
Although peptides C_{II}-11 and C_I-11 (peptides nearest the origin in fractions C_I-8 and C_{II}-8 in Fig. 14) do not have significantly different mobilities, the amino acid compositions are different. From the results of the amino acid analyses shown in Table 9 it is evident that peptide C_I-11 contains one less lysine than peptide C_{II}-11. In addition, peptide C_I-11 contains an odd amino acid designated as Lys-X. Lys-X has a mobility slightly greater than arginine as shown in Fig. 15. On the basis of its mobility Lys-X is a basic amino acid, but it is not one of the 20 common amino acids.

A qualitative amino acid analysis of C_I chymotryptic peptides by paper electrophoresis revealed that none of the other lysine residues of the protein were altered. As discussed for C_{II}, peptide T-8 was not recovered from the C_I peptide column. This is unfortunate since this peptide extends from residues 56 to 72. The two lysines in peptide C-11 (the peptide containing Lys-X) occur at residues 72 and 73.(see Fig. 10). An amino acid analysis of peptide T-8 would have determined the position of Lys-X.

That Lys-X does occur at residue 72 comes from two different kinds of evidence. In tryptic digests of C_I free lysine was recovered but not free Lys-X. Since lysine occurs at adjacent residues 72 and 73, trypsin should cleave on both sides of the latter amino acid. If Lys-X occurs at residue 73, it should be found as a free amino acid assuming, of course, Lys-X is a trypsin substrate. Assuming, however, that Lys-X is not a trypsin substrate and it occupies residue 73, it should be attached to peptide T-9, which it is not. These negative results argue, then, that Lys-X is not at residue 73. This is substantiated from an amino acid

TABLE 9

AMINO ACID COMPOSITION OF PEPTIDE C_I-11 AND C_{II}-11

Amino Acid	Molar Ratio		
	C_I-11	C_{II}-11	[1]Smith
Lysine	1.2	1.7	2
Lysine X	1.0	0	0
Leucine	1.0	1.0	1
Glutamic acid	1.7	1.8	2
Aspartic acid + Tyrosine	2.4	2.5	3

The peptides were purified by pH 6 electrophoresis. Amino acid analysis was carried out on paper using the cadmium-ninhydrin stain (METHODS). [1]These values were determined by Heller and Smith for the same peptide obtained from a chymotryptic hydrolysate of C_I (59).

analysis of peptides obtained from a pronase digestion of peptide C_I-11.
A peptide with the composition of (Asp, Lys-X) was recovered. Assuming
that proline is also present (proline develops as a pale yellow color
with ninhydrin and is often difficult to detect) this result suggests
that Lys-X is at residue 72 since aspartic acid and proline occur at
residues 70 and 71 respectively (Fig. 10). This conclusion is somewhat
tentative since peptides were recovered in low yields. Consequently,
the whole sequence of the original peptide, C_I-11, could not be accounted
for in the pronase digestion.

Identity of Lys-X An attempt was made to identify Lys-X by compar-
ing its mobility with that of several commercially available lysine de-
rivatives in the pH 1.9 electrophoresis system used for amino acid analy-
sis. D,L-allo-δ-hydroxylysine (Mann Research Laboratories), ε-N-methyl-
L-lysine (Fox Chemical Co.) and ε-N-dimethyl-L-lysine (Fox Chemical Co.)
were run along side standard amino acid mixtures. Dimethyl lysine and
hydroxylysine have mobilities comparable to Lys-X but the mobility of
methyl lysine is faster (compare Fig. 15b with Fig. 15a). This result,
then, only indicates that lysine can be modified in several ways to
result in a compound with a mobility similar to that of Lys-X.

Lysine Labeling of Lys-X The only difference in primary structure
between C_I and C_{II} is at residue 72. If this result is to be compatible
with the idea that C_{II} is a precursor of C_I, the lysine (residue 72) in
C_{II} must be converted to Lys-X in C_I. In other words Lys-X must be a
lysine derivative.

To determine if Lys-X is a lysine derivative, the following experiment
was carried out. Poky (137 g. wet weight) was pulsed for 60 min. with

Figure 15 a. Amino acid composition of peptides C_I-11 and C_{II}-11. Both peptides were purified by electrophoresis at pH 6.0. After acid hydrolysis, the amino acids were separated by electrophoresis at pH 1.9 for 1.75 hrs. at 40 volts/cm. The papers were stained with the cadmium-ninhydrin reagent (Table 1). The anode is on the left, the cathode on the right. Standard mixtures of amino acids were run along side each sample. From left to right the amino acids are: Lys, Arg, His, Amino ethyl Cys, Gly, Ala, Val, Ser, Ile, Leu, Thr, Met, Glu, Phe,(Asp,Tyr) and Tryp.

Figure 15 b. Electrophoretic mobility of hydroxylysine (HO-Lys), ε-N-methyl-lysine(Me-Lys) and ε-N-dimethyl-lysine (DiMe-Lys). Electrophoresis of these compounds was carried out at pH 1.9 under the same conditions as in Fig. 15 a. The unmarked samples are the same mixture of amino acids as in Fig. 15 a.

a

STD
C_I-11
C_{II}-11
STD

b

DiMe-Lys

Me-Lys

HO-Lys
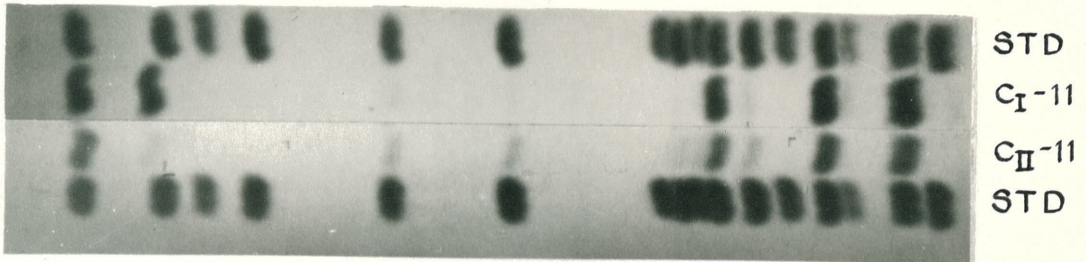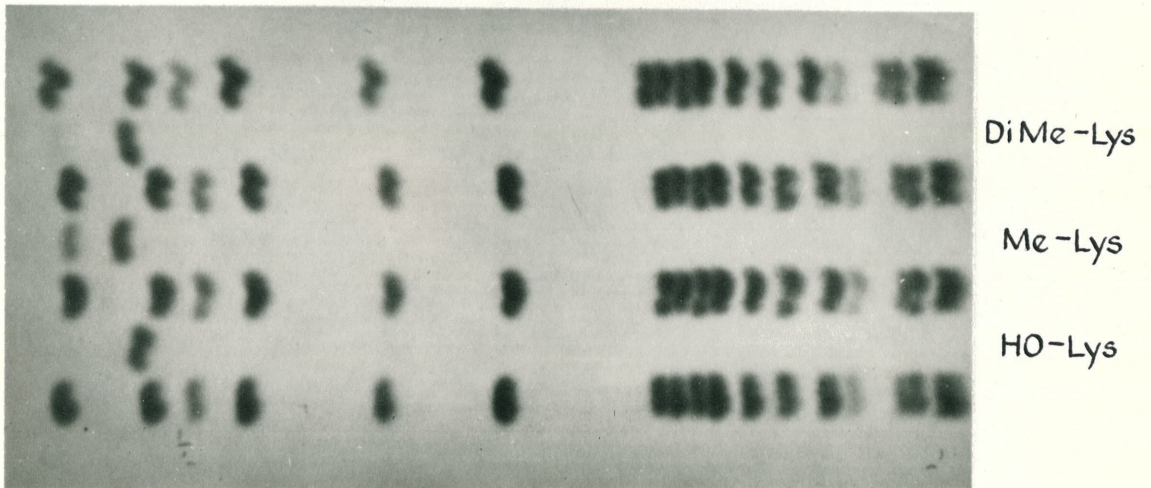
1 mc of D, L-lysine 4, 5-H^3 (New England Nuclear). Purified C_I (Fig. 16) from this sample was hydrolyzed with chymotrypsin. The separation and purification of the resulting peptides were carried out as described (see p. 83). The specific activity of amino acids from each peptide was determined (METHODS).

As a control, the radioactivity of all 18 different amino acids except tryptophan and cysteine was measured to verify that the lysine-H^3 had not randomized. The results are given in Table 10. Only those amino acids that occur once in a peptide are listed. Also the results for peptide C_I-11 are given in Table 11 as a representative sample of data.

As can be seen from Tables 10 and 11 only lysine and Lys-X had radioactivity significantly above background. The average specific activity of lysine was 5.3$^{\pm}$ 0.2 and the specific activity of Lys-X was 5.3. The specific activities of all other amino acids were less than 0.60. Since the specific activity of Lys-X is comparable to the average value calculated for lysine it can be concluded that Lys-X is a lysine derivative. This result confirms the earlier results based on physiological and chemical evidence that C_I is a derivative of C_{II}.

Distribution of C_I and C_{II} Between Mitochondria and Cytoplasm of Poky

As previously discussed, over half of the cytochrome c in young poky is not bound to mitochondria (7). The appearance of C_I in poky coincides with the decrease in cytochrome c content and presumably a binding of cytochrome c to mitochondria. The experiment described below was carried out to determine if one of the two cytochromes is preferentially bound to mitochondria in vivo.

Figure 16. Elution of C_I and C_{II} labeled with D, L-lysine-4,5-H^3 from a 2 x 50 cm. Amberlite CG-50 column by means of a linear gradient of NaCl. The cytochrome c concentration of each fraction was determined after reduction with sodium dithionite. An aliquot of 0.2 ml. was removed from each fraction for H^3 determination (METHODS). Fractions of C_I (60 to 80) were pooled.
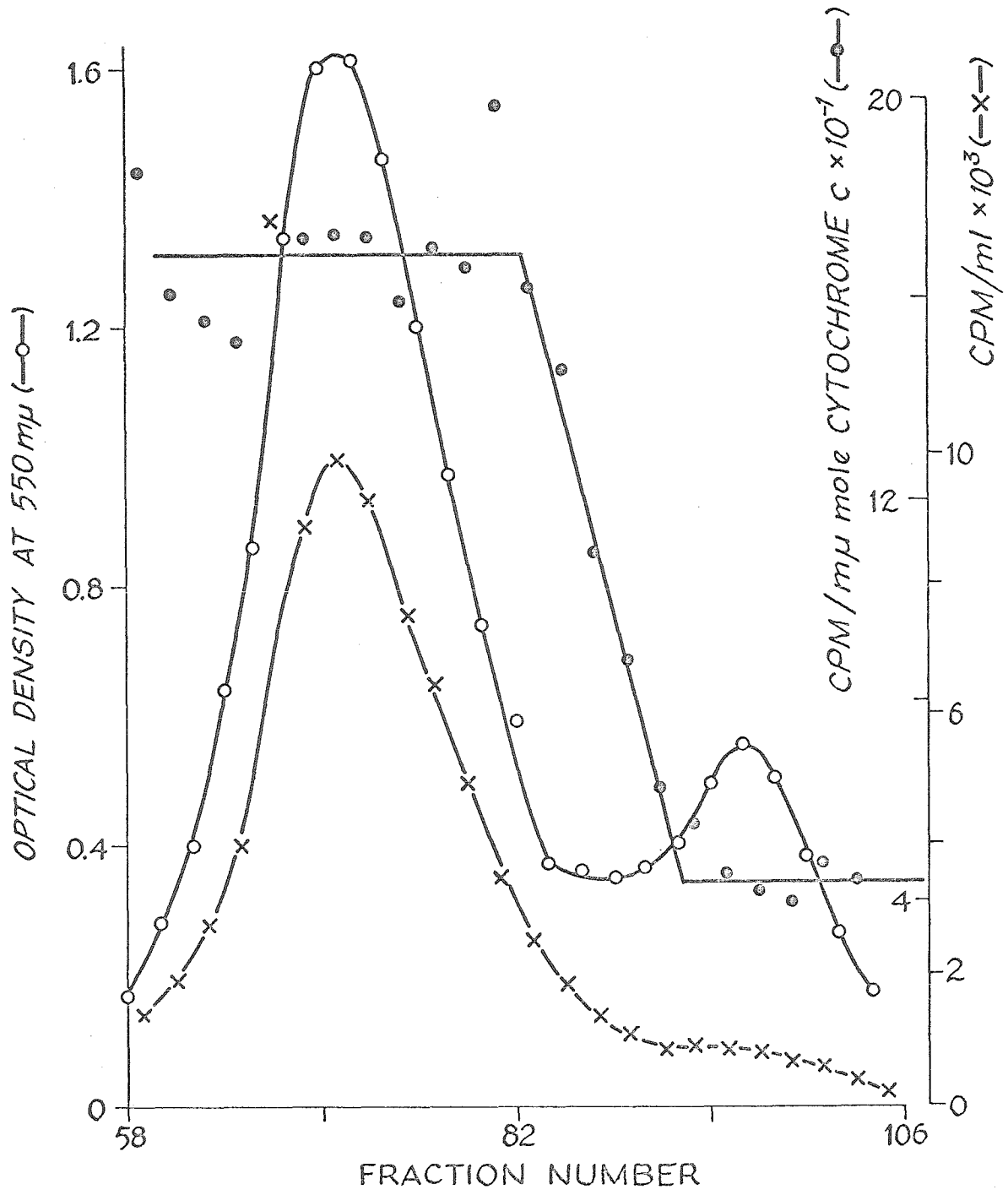
Table 10. Chymotryptic peptides of C_I were purified by paper electro

phoresis at pH 6.0. Amino acid analyses of each peptide were carried

out by paper electrophoresis after each acid hydrolysis. Amounts of

each amino acid were estimated after staining with cadmium ninhydrin.

The methanol extract of the paper containing the ninhydrin color was

used to determine the amount of radioactivity in each amino acid.

Approximately 30% of the radioactivity was eluted by this procedure.

The values are reported as the amount of radioactivity in the methanol

extract.

TABLE 10

DISTRIBUTION OF LYSINE-H^3 AMONG THE AMINO ACIDS OF C_I

Residue	Amino Acid	Specific Activity (cpm/mμ mole)
11	Lys	6.52
15	Ala	0.59
18	His	0.53
27	Lys	5.48
28	Ile	0.02
29	Gly	0.12
32	Leu	0.08
38	Arg	0.15
39	Lys	5.18
40	Thr	0.04
42	Ser	0.12
43	Val	0.05
44 and 46	Asp + Tyr	0.17
55	Lys	5.25
71	Pro	0.06
72	Lys-X	5.3
73	Lys	5.8
98	Met	0.05
99	Lys	4.62
100	Glu	0.17

A 40 g. sample of 50 hr. poky was divided into equal parts. Mitochondria were isolated from one part and purified by isopynic centrifugation in sucrose gradients (Methods). Cytochrome c was extracted at pH 10.5 the chromatographed as usual. From the second 20 g. sample of poky total cytochrome c was extracted then chromatographed. The ratio of C_{II} and C_I of the cytochrome c extracted from the whole cell was 0.55. Only C_I, however, was detected in mitochondria.

This result implies that the modification of lysine at residue 72 is the result of, or a prerequisite to, binding of cytochrome c to mitochondria in Neurospora.

Effect of High Concentrations of Chloramphenicol on Neurospora

Recent reports have indicated that yeast grown in high concentrations of chloramphenicol (0.5 - 4.0 mg./ml. of medium) exhibit characteristics of the respiration deficient petite mutants, i.e. no cytochromes a and b (27,28). Since the petite phenotype is similar to that of the poky mutant in Neurospora, it was of interest to determine if Neurospora grown in similar concentrations of chloramphenicol exhibits a poky phenotype.

In initial experiments the growth of wild type 4A in liquid cultures containing 4 mg./ml. of chloramphenicol was found to be extremely slow and variable. After 7 to 8 days of growth 10% of the cultures yielded an average of 20 mg. of mycelium while the remaining flasks exhibited no visible growth (cultures without chloramphenicol yielded from 80 to 100 mg. of mycelium after 3 days). The cytochrome content of the chloramphenicol treated mycelium was examined with the aid of a handspectroscope. All samples had a poky phenotype, i.e. excess cytochrome c and no cyto-

TABLE 11

DISTRIBUTION OF LYSINE-H^3 AMONG THE AMINO ACIDS OF PEPTIDE C_I-11

Amino Acid	Counts/Min.	mμ Moles	Specific Activity counts/min./mμ mole
Lysine	345	59.5	5.8
Lysine-X	307	57.8	5.3
Leucine	4.8	62.4	0.08
Glutamic acid	3.0	125.2	0.02
Aspartic acid + Tyrosine	7.4	137.8	0.02

Peptide C_I-11 was purified by pH 6.0 electrophoresis. Amino acid analysis was carried out by paper electrophoresis using the cadmium-ninhydrin stain Radioactivity was determined in the methanol extract of the paper. Approximately 30% of the radioactivity was eluted from the paper by this method. The number of counts reported are that amount in the methanol extract.

chromes a and b. However, when small amounts of the drug treated my-
celium were inoculated into medium without chloramphenicol the mold be-
haved as wild type in both growth tests and cytochrome content. Wild
type Neurospora exhibited the poky phenotype only in the presence of
chloramphenicol. Poky did not show any growth in chloramphenicol
medium (4 mg./ml.) up to 14 days.

Mycelium from wild type cultures grown in chloramphenicol was
transferred to slants of complete medium without the drug. Conidia from
these isolates were reinoculated into chloramphenicol medium. This
process was repeated three times. The resulting strains exhibited some
resistance to chloramphenicol. Growth was found to be more uniform than
that of wild type; all flasks inoculated with these strains exhibited
growth after about 3-4 days. The yield of mycelium from these resistant
strains at 8 days was higher than that of wild type grown in the drug
usually by a factor of 10 to 20.

One chloramphenicol resistant strain, CAP-1, was selected for fur-
ther study. The effects of varying concentrations of chloramphenicol
(1-4 mg./ml. of medium) on CAP-1 and wild type were compared by measuring
weights of mycelium and cytochrome content of the mycelium with the aid
of a Zeiss low-dispersion spectroscope. The results are shown in Table 12.
From 1 to 3 mg./ml. of chloramphenicol the growth of CAP-1 and wild type
are inhibited to the same extent. Similarly chloramphenicol has a
definite effect on the phenotype of both strains. At all concentrations
of chloramphenicol except 1 mg./ml., the growing mycelium exhibited a
characteristic poky cytochrome spectrum, i.e. no cytochromes a and b
and an accumulation of cytochrome c. However, the phenotype is variable,

TABLE 12

EFFECT OF DIFFERENT CONCENTRATIONS OF CHLORAMPHENICOL ON CAP-1

AND WILD TYPE

Hours of Growth	Concentration of Chloramphenicol (mg./ml.)	Wild Mycelial Dry Weights (mg.)	CAP-1	Phenotype
54.5	0	75	78	wild
	1	76	72	wild
	2	41	30	poky
	3	0+	0+	-
	4	0	0	-
82	0	125	120	wild
	1	129	110	wild
	2	84	84	wild
	3	47	55	poky
	4	0	0	-
198	4	8	60	poky

Cultures were grown for the indicated period of time at 25°C on a reciprocal shaker in 125 ml. flasks containing 20 ml. of Vogel's medium N plus 2% sucrose. After harvesting, a few grains of sodium dithionite were added to each pad of mycelium. The phenotype of the mycelium was ascertained by examination of the absorption bands of cytochrome a, b and c with a Zeiss low dispersion spectroscope. The above values are from one flask.

the cultures grown in 2 mg./ml. of the drug were phenotypically poky

at 43 hrs. but had a wild type cytochrome content at 75 hrs. It should

be emphasized that the difference in response of CAP-1 and wild type to

chloramphenicol is obtained only at a concentration of 4 mg./ml. of the

drug and, then, only after 7 to 8 days of growth.

To quantitate the difference between cells grown in the presence and

absence of chloramphenicol, mitochondria were isolated from CAP-1 grown

in Vogel's medium and Vogel's medium with 4 mg./ml. chloramphenicol.

Difference spectra of these mitochondria were compared with spectra of

mitochondria isolated from wild type, 4A, and poky grown in Vogel's

medium. The results are shown in Fig. 17a. The spectra of mitochondria

isolated from wild type and CAP-1 have absorption maxima at 605 and 550 mµ

and a shoulder at 560 mµ corresponding to cytochromes a, c and b respectively.

The difference spectra of particles prepared from poky and CAP-1 (grown in

the presence of the drug) however, lack the cytochrome a band and the cyto-

chrome b shoulder at 560 mµ. Since the cytochrome b band is somewhat

obscured, the spectra of the CAP-1 and CAP-1 chloramphenicol treated

mitochondria were determined by expanding the wavelength scale (Fig. 17b).

The cytochrome b band is clearly present in CAP-1 but not in CAP-1 grown

in the presence of chloramphenicol.

Since chloramphenicol produces the same effect as the poky phenotype,

the question of whether chloramphenicol resistance is controlled by nuclear

or cytoplasmic genes is of interest. A cross was made between CAP-1 as

the protoperithecial parent and wild type 25a. Ascospores were collected

as ordered tetrads from 3 separate perithecia by Larry G. Williams. Re-

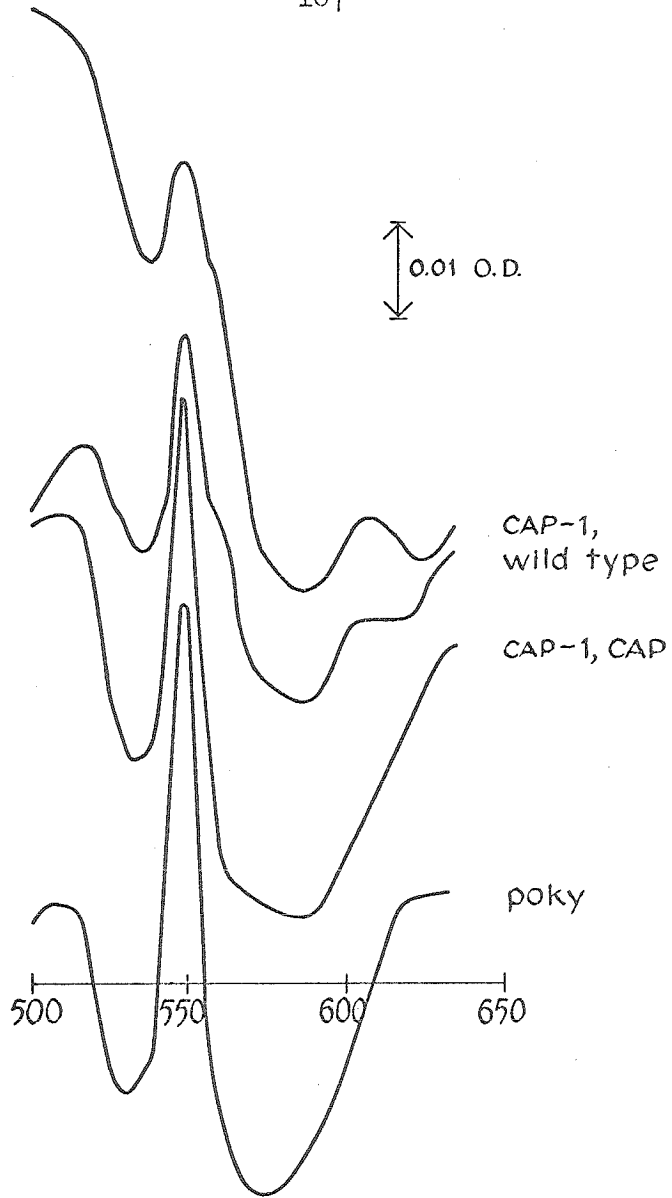sistance and sensitive strains were scored by dry weights after 8 days

Figure 17 a. Difference spectra of isolated mitochondria recorded at room temperature. Mitochondria were isolated by grinding cells in a mortar at 0°C with 2.5 parts of 0.25 $\underline{M}$ sucrose - 0.05 M phosphate buffer (pH 7.0) and 0.5 parts of sand per weight of tissue. Cell debris was removed by centrifugation at 1,000 x g for 10 min. The precipitate was washed once with the sucrose-phosphate buffer. The combined wash and 1,000 x g supernatant were centrifuged at 15,000 x g for 30 min. to obtain a mitochondrial pellet. After washing the pellet, the mitochondria were suspended in 0.5 ml. of the sucrose-phosphate buffer per g. wet weight of the original tissue. For determination of difference spectra, 0.1 ml. of the mitochondrial suspension was added to the reference and sample cells. The other ingredients of the assay mixture are those of Williams (71). The spectra were recorded by a Cary Model 15 recording spectrophotometer using a 0 to 0.1 absorbance slidewire.

The abbreviations designate the strain from which the mitochondria were isolated. CAP-1, CAP denotes mitochondria isolated from CAP-1 grown in Vogel's medium containing chloramphenicol (4 mg./ml.). All other strains from which mitochondria were isolated were grown in Vogel's medium without the drug.

Figure 17 b. Spectra of CAP-1, CAP and CAP-1 mitochondria isolated as described in Fig. 17 a. The wavelength scale was expanded and a 0 to 1.0 absorbance slidewire was employed to demonstrate the absence and presence of the cytochrome b shoulder at 560 mμ in CAP-1, CAP and CAP respectively.

a

0.01 O.D.

CAP-1,
wild type

CAP-1, CAP

poky

500   550   600   650

b

0.1 O.D.

CAP-1, CAP

CAP-1

540   560   580
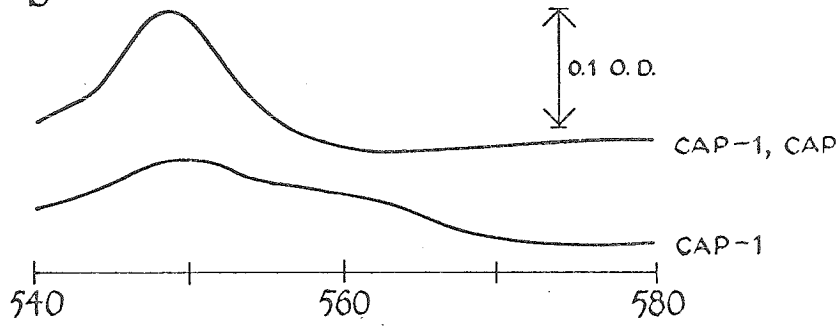
growth in 4 mg./ml. of chloramphenicol. Because of the variable growth

of wild type strains in this concentration of chloramphenicol three

separate flasks were inoculated with conidia from cultures derived

from each ascospore. The yields are reported as the combined weights

of mycelium from the three flasks. The results in Table 13 indicate

that the chloramphenicol resistance is gene determined and segregates in

a Mendelian fashion. The resistance, however, does not appear to be

the result of single Mendelian gene since at least two levels of re-

sistance appear to be segregating.

TABLE 13

ANALYSIS OF CROSS OF CAP-1 X 25a (WILD)

PERITHECIUM NO.	1			2			3
ASCUS SPORE PAIR	MYCELIAL DRY WEIGHTS (MG)						
1	64	23	22	4	14	13	176
2	29		2	4	0	85	36
3	26	49	2	111	162	0	5
4	4	41	4	105	33	43	4
CAP-1	170						
4A,WILD	10						

Values are from three flasks. Dissected ascospores were germinated by heat shock at 60°C for 30 min. Each ascospore was transferred to a slant of minimal medium. Conidia from each of the resulting cultures was inoculated into three separate 125 ml. flasks containing 20 ml. Vogel's medium N plus 2% sucrose. Mycelia from the flasks were collected after 198 hrs. of growth at 25°C on a reciprocal shaker.

DISCUSSION

The results which are presented here demonstrate that Neurospora crassa synthesizes two chromatographically separate cytochromes c, C_I and C_{II}, which differ in structure by a secondary modification at residue 72 in the primary amino acid sequence. A precursor-product relationship was established between the two proteins: C_{II} is converted to C_I by modifying the lysine at residue 72 to Lys-X. The kinetics of synthesis of the two proteins were studied in poky then compared to those of wild type and the respiration deficient mutants, mi-3, cyt-1 and pof. Young poky was found to accumulate C_{II} and to have a delayed synthesis of C_I as compared to wild type. On the other hand, both mi-3 and cyt-1 exhibit normal kinetics of C_I and C_{II} synthesis while those of pof are intermediate between wild type and poky. All three strains, however, have an excess of cytochrome c as does poky.

Some controversy has centered around the question of whether Neurospora contains more than one cytochrome c. Hardesty (7) first isolated the protein from wild type and the respiration deficient mutants; poky, pof, and mi-3, by alkali extraction. Pure preparations of poky cytochrome c exhibited three electrophoretic components in the free boundary and continuous flow systems. Similarly, cytochrome c extracted from all of the above strains was heterogeneous on starch block electrophoresis. Although the protein smeared, two to three components could be discerned in the cytochrome c from each strain. The poky and wild type cytochromes c, however, were found by Hardesty to have identical sedimentation constants. The heterogeneous electrophoresis patterns can be partially explained by the observation that the poky cytochrome c preparation, although

thought to contain only oxidized cytochrome c, was found to be contaminated by significant amounts of the reduced protein. The oxidized and reduced forms of cytochrome c are known to be electrophoretically separable (1).

Heller and Smith (95) also compared the electrophoretic behavior of wild type and poky cytochrome c obtained by base extraction. Both cytochromes c exhibited single bands with identical mobilities at pH 8.5 and 4.7 on cellulose acetate at pH 8.3 and 4.3 on acrylamide gel. From these results and total amino acid compositions of both proteins, they concluded that Neurospora synthesizes one cytochrome c and that the poky and wild type proteins are identical.

The results of the present study are in agreement with those of Hardesty. Two chromatographically separable cytochromes c were found in wild type and several respiration-deficient strains. The temporal separation of C_I and C_{II} synthesis in poky (Fig. 5) explains the discrepancy between these results and those of Heller and Smith (95). These authors extracted cytochrome c from poky that was physiologically quite old. They obtained an average yield of 75 g. wet weight of tissue per 15 l. of medium. In the present experiments the yield of 40 hr. poky, an age when C_I and C_{II} occur in approximately equal concentrations, was 0.9 g. wet weight of tissue per 15 l. of medium. It is, therefore, not surprising that Heller and Smith only found C_I since C_{II} is present in young poky only.

C_I and C_{II} were first detected in poky. However, the two cytochromes c are not the result of a poky mutation since both proteins were

found in wild type and the other respiration deficient strains. A comparison of the amounts of C_I and C_{II} in extracts from different ages of poky and wild type revealed distinctive differences between the two strains (Fig. 6). Compared to the normal situation, young poky accumulates C_{II} and has a delayed synthesis of C_I (Figs. 5 and 6). In poky, the conversion of C_{II} to C_I parallels the decrease of the cytochrome c content and the change of the mutant phenotype to a more normal state. The decrease in cytochrome c content was shown to result from a decreased synthesis and not from degradation. This is consistent with the idea that, as the poky phenotype becomes more normal, more of the cytochrome c is bound to mitochondria . In young poky, Hardesty found 67 per cent of the cytochrome c unbound and only 10 per cent in the mitochondrial fraction. Unfortunately the intracellular distribution of cytochrome c has not been determined in older poky.

To understand the significance of the two cytochromes c and these events in poky, it is important ot elucidate the exact chemical nature of the modification of the lysine at residue 72 that results in the conversion of C_{II} (lysine) to C_I (Lys-X). That only C_J was found in isolated poky mitochondria, even though the whole cell contains both proteins, may provide a clue to the significance of the difference between C_I and C_{II}. The conversion of C_{II} to C_I may reflect a binding of cytochrome c to mitochondria. The finding of C_I in poky mitochondria indicates that this protein is preferentially bound at one age of the mutant only. C_{II} is undoubtly bound to mitochondria in germinating poky conidia since this protein was found to constitute the whole cytochrome c complement at this age (Fig. 5). It is plausible, however, that low amounts of C_I

(10 per cent or less) were obscured in the chromatographic elution profiles.

The position of Lys-X (residue 72) in the amino acid sequence of cytochrome c is significant. The amino acid sequence from residues 72 to 80 has been strictly conserved throughout the twenty-five mammalian type cytochromes c that have been sequenced (57). This segment is thought to be important in hemochrome formation and to be the area of interaction with another protein (INTRODUCTION). However, the nature of the interaction and the exact species to which cytochrome c complexes in vivo is unknown. Takemori et al.(94) have suggested an interaction between the formyl group of cytochrome a heme and an ϵ-amino group in cytochrome c to form a Schiff's base (analogous to the reaction of pyridoxal phosphate catalyzed reactions (96)). This idea was substantiated by the finding that chemical modification of lysine at residue 72 or 73 in cytochrome c causes the protein to exhibit a 50 per cent loss of activity in the cytochrome oxidase assay (65). The loss of cytochrome c reactivity, it should be noted, could also be explained as change in cytochrome c conformation due to the large size of the entering group (trinitrophenyl sulfonate). Cytochrome c has also been shown to bind with mitochondrial structural protein (MSP) in vitro. Edwards and Criddle (21) have detected 1:1 complex between cytochrome c and MSP by fluorescence quenching. The formation of the complex is sensitive to salt and, therefore, is ionic in nature. Again since cytochrome c contains a preponderance of lysine residues, whose side chains are thought to extend outward from the molecule (66), one or more of these residues could be involved in this binding. A binding study of trinitrophenylated cytochrome c with

mitochondrial structural protein would provide some interesting results in this regard.

The temporal separation of the synthesis of C_I and C_{II} in Neurospora is reminiscent of the iso-cytochromes of yeast (50). Slonimski et al.(54) (see INTRODUCTION) have shown that the synthesis of iso-2-cytochrome c is immediate and rapid followed by the synthesis of iso-1-cytochrome c at a slower rate after induction with oxygen. In both Neurospora and yeast the more basic cytochrome is synthesized first. However, the similarity ends here. The iso-cytochromes c of yeast are encoded by separate structural genes(2) and are known to differ by thirteen amino acid residues (51). The Neurospora cytochromes c, on the other hand, differ by a secondary modification of lysine at residue 72 and for this reason are undoubtly encoded by a single cytochrome c structural gene. In addition, kinetics of C^{14} lysine incorporation into C_I and C_{II} and long term pulse-labeling experiments demonstrated a precursor-product relationship between the two cytochromes c in Neurospora. The experiments with acti-dione inhibition of cytochrome c synthesis further emphasize the difference between the two systems. Low levels of acti-dione (0.2µg./ml.) completely inhibit the synthesis of both cytochromes c in poky although the conversion of C_{II} to C_I is unaffected. In yeast, however, acti-dione (25µg./ml.) inhibits the oxygen-induced synthesis of iso-1 although the synthesis of iso-2 is inhibited by only 30 to 40 per cent (55).

The growth and phenotype of wild type Neurospora are severely affected by high concentrations of chloramphenicol. The formation of mitochondrial cytochromes b and a is inhibited (Fig. 17) while

cytochrome c is synthesized in excess. High concentrations of the drug
(4mg./ml.) inhibit the growth of Neurospora. However, since 10 per cent
of the cultures inoculated with wild type conidia grow very slowly, a
small portion of the conidial population must be resistant to the drug.
Similarly, Linnane et al. (27,28,92) also have shown that chloramphenicol
inhibits the synthesis of cytochromes a, a_3, b and c_1 in aerobically
growing yeast cells, although cytochrome c synthesis is unimpaired. The
growth of yeast, however, is not affected by the drug provided that
high concentrations of a fermentable substrate are present.

Chloramphenicol has been shown to inhibit protein synthesis in
mitochondria isolated from yeast and mammalian cells but does not affect
amino acid incorporation by cytoplasmic ribosomes from either source
(30,31,32). In bacterial protein synthesizing systems (in vitro),
which are inhibited by chloramphenicol, Vazquez (97) has found that the
drug binds to the 50S subunit of the ribosomes although the binding is
not affected by the presence of the messenger RNA. The effect of the
drug seems to be at the stage of peptide bond formation since there is
a shift from the synthesis of long chains to di- and tripolypeptides in
its presence (98).

Based on these observations and the effect of chloramphenicol on
the yeast phenotype, Linnane has concluded that the drug specifically
inhibits mitochondrial protein synthesis in this organism. The same
conclusion may also be true for Neurospora. The lack of cytochromes a
and b plus the excessive amounts of cytochrome c found in wild type grown
in high concentrations of chloramphenicol are similar to the poky pheno-
type (12). The effect of the drug on Neurospora, therefore, is interesting

since it is similar to that of a maternally inherited mutation. The
poky mutation results in an amino acid substitution in the mitochondrial
structural protein (MSP) whose primary structure is undoubtedly encoded
by mitochondrial DNA (17). Presumably, then, an inhibition of MSP syn-
thesis by chloramphenicol will result in a poky-like phenotype. By this
argument, reduced amounts of MSP in Neurospora have an effect similar
to the Tryp→Cys substitution in MSP resulting from the poky mutation.
Since the resistance of Neurospora to chloramphenicol is determined by
nuclear genes (Table 13), one would assume that the component(s) of mito-
chondrial ribosomes to which chloramphenicol binds are not encoded by
mitochondrial DNA.

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