Enzyme Induction in *Neurospora crassa*

Thesis by

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In Partial Fulfillment of the Requirements for the

Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1966

(Submitted June 23, 1965)

ACKNOWLEDGEMENTS

I wish to thank Dr. Norman H. Horowitz for his sustaining guidance and understanding criticism during the course of this work. The help of all the members of his group is greatly appreciated. I also thank all the other members of the Biology Division who helped to make my years here so pleasant and rewarding.

This work was partially supported by a Public Health Service Training Grant (2G-86) and by fellowships from the Woodrow Wilson Foundation and the National Science Foundation.

My deepest appreciation goes to my wife, Ann, whose patience and love helped bring this thesis to completion.

ABSTRACT

I. Studies on Nicotinamide Adenine Dinucleotide Glycohydrase (NADase)

NADase, like tyrosinase and L-amino acid oxidase, is not present in two day old cultures of wild type Neurospora, but is coinduced with those two enzymes during starvation in phosphate buffer. The induction of NADase, like tyrosinase, is inhibited by puromycin. The induction of all three enzymes is inhibited by actinomycin D. These results suggest that NADase is synthesized de novo during induction as has been shown directly for tyrosinase. NADase induction differs in being inhibited by certain amino acids.

The tyrosinaseless mutant ty-1 contains a non-dialyzable, heat labile inhibitor of NADase. A new mutant, P110A, synthesizes NADase and L-amino acid oxidase while growing. A second strain, pe, fl;cot, makes NADase while growing. Both strains can be induced to make the other enzymes. These two strains prove that the control of these three enzymes is divisible. The strain P110A makes NADase even when grown in the presence of Tween 80. The synthesis of both NADase and L-amino acid oxidase by P110A is suppressed by complete medium. The theory of control of the synthesis of the enzymes is discussed.

II. Studies with EDTA

Neurospora tyrosinase contains copper but, unlike otherphenol oxidases, this copper has never been removed reversibly. It was thought that the apo-enzyme might be made in vivo in the absence of copper. Therefore cultures were treated with EDTA to remove copper before the enzyme was induced. Although no apo-tyrosinase was detected, new information on the induction process was obtained.

A treatment of Neurospora with 0.5% EDTA pH 6, inhibits the subsequent induction during starvation in phosphate buffer of tyrosinase, L-amino acid oxidase and NADase. The inhibition of tyrosinase and L-amino acid oxidase induction is completely reversed by adding 5×10^{-5}M $CaCl_2$, 5×10^{-4}M $CuSO_4$, and a mixture of L-amino acids (2×10^{-3}M each) to the buffer. Tyrosinase induction is also fully restored by 5×10^{-4}M $CaCl_2$ and amino acids. As yet NADase has been only partially restored.

The copper probably acts by sequestering EDTA left in the mycelium and may be replaced by nickel. The EDTA apparently removes some calcium from the mycelium, which the added calcium replaces. Magnesium cannot replace calcium. The amino acids probably replace endogenous amino acids lost to the buffer after the EDTA treatment.

The EDTA treatment also increases permeability, thereby increasing the sensitivity of induction to inhibition by actinomycin D and allowing cell contents to be lost to the induction buffer. EDTA treatment also inhibits the uptake of exogenous amino acids and their incorporation into proteins.

The lag period that precedes the first appearance of tyrosinase is demonstrated to be a separate dynamic phase of induction. It requires oxygen. It is inhibited by EDTA, but can be completed after EDTA treatment in the presence of 5×10^{-5}M $CaCl_2$ alone, although no tyrosinase is synthesized under those conditions.

The time course of induction has an early exponential phase suggesting an autocatalytic mechanism of induction.

The mode of action of EDTA, the process of induction and the kinetics of induction are discussed.

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INTRODUCTION

How is a gene turned on and off? This is one of the basic unanswered questions of biochemical genetics. Two clear examples will illustrate that genes are indeed turned on and off during development and differentiation. First is the case of hemoglobins A and F (1). Hemoglobin A is the major hemoglobin in the human adult. Hemoglobin F is the major hemoglobin in the human foetus. These differ in that Hb A has the structure $\alpha_2\beta_2$ while Hb F has the structure $\alpha_2\gamma_2$. The γ and β peptides have 42 amino acid differences (2) and are specified by different structural genes. Since hemoglobin F is not detected in normal human adult blood (1), the γ chain gene is turned off in adults. Cook et al. (3) have estimated that Hb A first appears at the 34th week of gestation, that is, the β chain's structural gene is turned on at that time.

Second is the case of nuclear transplantation in amphibians. Gurdon (4) showed that when a nucleus from fully differentiated intestinal cells of a feeding Xenopus tadpole was put into an anucleate egg cell, a normal larva developed from some of the eggs. This proved that these nuclei contained all of the genetic information necessary to the development of a tadpole. But such nuclei, when in intestinal cells are not expressing all of this information, for example, they are not making lens protein or myelin or melanin.

Thus some genes are not turned on in intestinal cells which are turned on in other tissues, yet those genes are present in the intestinal cell. Thus it is clear that a basic aspect of differentiation is the control of gene action.

Jacob and Monod (5) have proposed the operon model as an explanation of the control of gene action in Salmonella and E. coli. This model proposes that there are two classes of genes: structural genes, which determine the primary sequence of a protein, and regulatory genes, which determine whether or not structural genes can function. It also assumes that the control exercised by regulatory genes is negative, i.e., they prevent protein synthesis rather than stimulate it. The structural genes which are to be regulated together are grouped in a single region of the genome, called an operon, which is turned on and off as a single unit. Further, the theory proposes that the product of the regulatory gene operates at the level of transcription of genes into messenger RNA and that this messenger RNA is short-lived. In the model, the regulatory gene makes a repressor. The repressor can combine with a special region of the operon, the operator region. When the repressor is combined with the operator region, no transcription of the structural genes in the operon can occur.

According to the Jacob and Monod theory, induction and repression are different sides of the same coin. During the induction of enzymes, e.g. β-galactosidase, the effector molecule, a β-galactoside, reacts with the repressor and thereby prevents the repressor from combining with the operator region. This permits the structural genes of the operon to be transcribed into messenger RNA. During feedback repression the effector molecule, e.g. histidine, reacts with the repressor, allowing it to combine with the operator region, thereby preventing the synthesis of the messenger RNA of the structural genes. This gives the cell control of the amount of histidine synthesized in the cell. When the histidine concentration is high, the enzymes which synthesize histidine are repressed, so less histidine is made. When the histidine concentration in the cell is low, the histidine enzymes are synthesized, so more histidine is made (6). Thus the cell can regulate its histidine synthesis to meet its demands.

A consequence of the operon theory is that induced cells should contain the messenger RNA corresponding to the operon, while uninduced cells should not. The operon theory received major confirmation when this prediction was verified (6, 7, 8, 9). A second consequence of the operon theory is coordinate control. That is, upon induction or repression, the amount of each enzyme of the

operon should increase by the same factor (5).

While minor changes have been necessary to accommodate new results, and the repressor molecule has not been isolated, the operon, essentially as proposed by Jacob and Monod, has been accepted by most geneticists as a valid description of the events occurring during induction and repression of enzymes in Salmonella and Escherichia coli.

As soon as the operon was proposed, people started looking for analogous control systems in higher organisms (1, 10, 11, 12, 13). However, there are differences between higher organisms and enterobacteria which suggest that the operon model must be modified before it can be applied to higher organisms. First, in higher organisms, such as yeast and Neurospora, the structural genes of a single biochemical pathway are usually scattered on many different chromosomes (16, 17). One example is the histidine pathway, whose biochemical reactions are identical in Salmonella, yeast and Neurospora (14). These genes form a classical operon in Salmonella (15), but are spread among several different chromosomes in Neurospora (16) and yeast (17). An even more extreme example is the case of proteins such as hemoglobin, where the structural genes for the different polypeptide chains are unlinked and yet the amount of the two chains made appears to be under coordinate control (1).

In both yeast and Neurospora, a new type of regulatory gene has been discovered, the ty-1 and ty-2 genes in Neurospora (18, 21) and the ga-4 gene in yeast (12). Mutations in these genes prevent the normal induction of the enzymes they control. These mutant genes are recessive to the wild type gene and are unlinked to the structural genes they regulate (12,18). No corresponding regulatory genes are known in bacteria, where one type of regulator mutation, the i^- type, causes constitutive production of the enzymes controlled, and the other type, the i^s type, prevents induction of the enzymes and is dominant (5).

A third way in which bacteria differ from higher organisms is the range over which the amount of an enzyme varies in cells. Thus in rat livers the amount of ornithine transcarbamylase varied five-fold with diet (79). In E. coli K12, the level of the homologous enzyme may vary 150-fold, depending on the amount of arginine in the growth medium (22). Cox and Macleod (81) have studied the regulation of alkaline phosphatase in human cell lines in tissue culture. Unlike E. coli, the amount of enzyme present is not dependent on the amount of inorganic phosphate present, but the enzyme can be induced with phenylphosphate. The greatest difference in enzyme concentration among all the cell lines studied was 100-fold. In certain cell lines in which the enzyme was inducible, the greatest increase due to induction was 25-fold. On the other hand, Garen and

Echols (82) find a 1000-fold increase in alkaline phosphatase activity when E. coli is switched from a high phosphate to a low phosphate medium.

A similar difference is found between bacteria and fungi. Thus Metzenberg (75) reported an 80-fold difference in invertase (β-fructofuranosidase) activity between Neurospora grown on mannose or on galactose. In E. coli, the induced level of β-galactosidase is more than 1000 times the activity in uninduced cells (5).

A few structural genes appear to be clustered in Neurospora and yeast. Thus Ahmed et al. (10) have suggested that histidine 3a of Neurospora may be analogous to a fragment of the histidine operon of Salmonella which has persisted in Neurospora. This has been challenged by Catcheside (11), who has suggested that the histidine 3a region codes for a single protein having at least two and possibly three enzymic functions. In yeast, the genes homologous to those which make up the galactose operon in E. coli are clustered, suggesting that they might be an operon (12).

In view of the general non-clustering of structural genes of biochemical pathways in higher organisms, it has been suggested that in higher organisms genes may be grouped on a different basis, such as when they are needed in differentiation--that is, as developmental operons rather than as biosynthetic operons (19, 13).

At the same time, we might use as a model for higher organisms those cases in the bacteria where the genes of a biochemical pathway are not clustered, for example, the arginine pathway.

The eight structural genes of the arginine pathway are scattered among five different sites on the bacterial chromosome (20). In repressible strains, the enzymes of the pathway are all specifically repressed by arginine (20, 21, 22). This control is parallel in that every enzyme is repressed by arginine, but the factor by which the amount of an enzyme changes is different for different enzymes. In contrast, the coordinate control in the histidine operon causes every enzyme in the operon to increase or decrease by the same factor as every other enzyme (26). Mutations have been found due to which arginine does not repress the synthesis of the eight enzymes. These mutations map at the R_{arg} locus (20, 21), and are recessive to the wild-type allele (24, 25). The arginine system is the first in which the repression of the entire pathway by the effector molecule (arginine) has broken down. Vogel et al. (20) have found a mutation affecting the fourth enzyme in the pathway in such a way that it is induced by arginine even while the other enzymes in the mutant are being repressed. The mutant enzyme formed differs qualitatively from the wild-type enzyme. A model proposed for the arginine system is the one-repressor many-operons model (25). In it each

structural gene or group of structural genes has its own operator region. The repressor molecule made by the R_{arg} gene supposedly reacts with these operators, turning their operons on and off together. Since different genes are under the control of different operators, the control is parallel rather than coordinate. In this model the mutant gene found by Vogel et al. (20) has an altered operator region in structural gene IV which combines with the R_{arg} gene product when arginine is absent rather than when it is present, so that the enzyme is formed when arginine is present.

Horowitz (13) has suggested that the Neurospora enzymes, tyrosinase(cf. Appendix 1) and L-amino acid oxidase (cf. Appendix 1) are under common control. Hirsch (83) has shown that tyrosinase plays an important role in sexual differentiation in Neurospora. Horowitz (13) suggested that both enzymes could be part of an operon of enzymes involved in sexual differentiation. Neither enzyme is present in rapidly growing vegetative wild type cultures. However, if growth is inhibited by starvation, then both enzymes are made. Similarly, on Westergaard-Mitchell crossing medium (90), which is nitrogen deficient, both enzymes are made during protoperithecial formation. Two mutants were found by Westergaard which were female sterile and did not make tyrosinase. Further investigation in this laboratory showed them to be two different unlinked recessive mutations, called tyrosinaseless-1 (ty-1)

and tyrosinaseless-2 (ty-2). Neither of these mutants makes tyrosinase when induced by starvation in phosphate buffer unless an inducer such as ethionine is present. Horowitz showed that ty-1 likewise made L-amino acid oxidase if ethionine was added to the buffer, but not in its absence. Thus the two enzymes do appear to be under common control. If they are part of the same operon, then their induction should be coordinate, i.e. they should always be present in the cell in the same ratio independent of the total amount of each enzyme present. Horowitz concluded that this was true in both ty-1 and wild type, so that they might be part of the same operon.

The enzyme NADase or DPNase (cf. Appendix 1) is found in large amounts in Neurospora when growth is limited by zinc and/or biotin (27). Zalokar and Cochrane (28) found that NADase is present in highest concentrations in conidia and is made in large amounts during conidiation. In this laboratory (29), NADase was found in cultures undergoing sexual differentiation on Westergaard-Mitchell medium and in extracts of cultures induced by starvation in phosphate buffer. These findings prompted us to investigate the control of NADase synthesis and its relationship to the control of tyrosinase and L-amino acid oxidase synthesis.

The first section of this thesis is a study of the production of NADase by Neurospora and its relation to the induction of tyrosinase and L-amino acid oxidase. The second section is a study of the effects of EDTA on Neurospora. This investigation started as an attempt to make apo-tyrosinase in vivo by removing the copper from an inducing culture with EDTA. Although the original objective was not attained, the experiment revealed some properties of the induction system which warranted further study.

MATERIALS AND METHODS

STRAINS

Wild type 69-1113a was used in most experiments. It produces large amounts of the thermostable form of tyrosinase upon induction (31, 32). In some early experiments wild type 120-1 T^{sing}, which makes the Singapore form of tyrosinase was used (30).

The wild type strain Scott A is a new strain of Neurospora crassa found in the San Gabriel Mountains of California by Mr. William Scott (63). It is reported by Mrs. Mary B. Mitchell to be more fertile than other wild types (33). Other wild types investigated were 1A, Abbott 4A, Abbott 12a, 25a, Em 5297a, and St. 74A.

The two tyrosinaseless mutants, ty-1 and ty-2, are female-sterile, recessive mutants that are unlinked to one another or to the structural locus for tyrosinase (18, 31). The mutant ty-1 carries the morphological marker velvet which has not been separated from the tyrosinaseless property. Neither mutant makes significant amounts of tyrosinase when induced in phosphate buffer unless an inducer is added to the buffer.

The strain pe, fl; cot (Y8743m, L; C102t) was obtained from Mrs. Joyce Maxwell of this laboratory.

The strain P110A was the generous gift of Dr. Dow Woodward of Stanford University, who found that it is constitutive for NADase.

P110A was selected for its poor growth on sucrose. It grows better when pyruvate is present as a supplementary carbon source.

MEDIA

Vogel's minimal medium N (34) was the only minimal medium used. The complete medium contained 1x Vogel's basal salts supplemented with 1.5% malt extract (Difco), 0.5% yeast extract (Difco), 0.025% casamino acids (Difco), and 2.0% glycerol. Solid media contained 1.5% agar (Difco).

Chemicals:

All chemicals used were reagent grade or the best grade available. Table sugar was used in the growth medium except in experiments where the constitution of the medium was critical.

GROWTH OF CULTURES AND INDUCTION OF THE ENZYMES

A few drops of a conidial suspension were inoculated into 20 ml of Vogel's liquid minimal medium N (34) in 125 ml Erlenmeyer flasks. The cultures were grown in the dark at 25°C without shaking. After 40-48 hours growth, the flasks were removed from the dark. In experiments with the slow-growing mutants pe, fl; cot and P110A, the cultures were grown for 3 or 4 days until the mycelial pad had attained a size which could be handled conveniently. The growth medium was decanted aseptically, leaving the mycelium on the side of the flask. About 10 ml of sterile water was added and

the pad carefully resuspended in it. This water was decanted and the water wash was repeated once. After the second rinse was decanted, 5.0 ml of sterile 0.02M sodium phosphate buffer pH 6.0 were added. This buffer was supplemented with any necessary additions such as cations, inducers, or inhibitors. Then the mycelium was resuspended in the buffer, and the cultures were returned to the dark at 25°C until they were harvested, usually two days later. The mycelium was harvested, after removing a sample of the induction buffer, by pouring it onto a Whatman #1 filter on a Büchner funnel under suction. The mycelium was weighed, wrapped in aluminum foil which was labelled, and stored at -15°C until it was ground and extracted. Samples of the growth medium or induction buffer were stored at -15°C and assayed for NADase.

THE TREATMENT WITH EDTA

Two-day-old cultures were washed (as outlined under induction) with a solution of 0.5% ethylenediaminetetraacetic acid disodium salt (EDTA) in 0.10M sodium phosphate buffer pH 6. In earlier experiments they were washed once. Most experiments involve two washings. The results obtained did not differ but were more easily reproduced when cultures were washed twice. After washing with EDTA, the cultures were washed once or twice with sterile water to remove the EDTA. Again, two washings gave more easily reproduced results and have been used in most experiments.

The washed mycelium was then suspended in 5 ml of induction buffer. The exact nature of the buffer varied in different experiments and is noted in each table. It usually consisted of 0.02M sodium phosphate pH 6.0 supplemented with cations and/or L-amino acids.

The mixture of L-amino acids contained the amino acids present in tyrosinase except tyrosine (to avoid melanization of the extracts). (Table 1)

Table 1. The Amino Acid Mixture Added to the Induction Buffer After EDTA Treatment.

L-Alanine	L-Lysine
L-Aspartic Acid	L-Methionine
L-Arginine	L-Phenylalanine
L-Glutamic Acid	L-Proline
L-Glycine	L-Serine
L-Histidine	L-Threonine
L-Isoleucine	L-Tryptophan
L-Leucine	L-Valine

All the amino acids were present in the same concentration which is listed for each experiment.

THE EXTRACTION OF THE ENZYMES

The mycelial pad was ground with sand in a cold mortar. Then cold .10M sodium phosphate buffer pH 6.0 was added, usually 10 or 20 parts by weight. The extract was centrifuged for 5 minutes at 10,000 xg to remove sand and cell debris and the supernatant was removed and stored frozen at -15°C until assayed.

In certain experiments crude extracts were dialyzed overnight at 4°C against two changes of0.10M sodium phosphate buffer pH 6.0.

THE ASSAY OF TYROSINASE ACTIVITY

Tyrosinase activity was determined on 0.01 to 0.50 ml aliquots of the extracts by the photometric method described previously (18). The amount of dopachrome formed from DL-dopa (cf. Appendix1)was measured in a Klett-Summerson colorimeter using a blue (#54) filter. The activities are expressed in Enzyme Commission units (84) per gram wet weight of the mycelium. One unit is the amount of enzyme which transforms one μ mole of L-dopa per minute under the assay conditions. The translation of absorbance measurements into Enzyme Commission units for the tyrosinase-dopa system has been explained previously (35).

THE ASSAY OF L-AMINO ACID OXIDASE ACTIVITY

L-Amino acid oxidase was assayed by measuring the rate of formation of phenylpyruvate from L-phenylalanine as described by Horowitz (13). The reaction mixture, containing 0.20 ml of crude extract and 2.05 ml of 0.10M sodium phosphate buffer pH 6,0, was equilibrated with oxygen in a Dubnoff metabolic shaking incubator at 35°C for 5 minutes. The reaction (cf. Appendix 1) was started by adding 0.25 ml of 0.01M L-phenylalanine. The reaction was

stopped after 20 minutes by immersing the reaction mixture in boiling water for 5 minutes. The mixture was centrifuged to remove the coagulated proteins, and phenylpyruvate was determined in the supernatant by the ferric chloride method of Jervis (36). Alternatively, the reaction was stopped by adding 1.0 ml of 20% metaphosphoric acid to the reaction mixture. The precipitate was removed by centrifugation and the phenylpyruvate determined by the enol-borate method of Lin et al. (37).

The second method is more convenient and has been used in most of the experiments reported in this thesis. The millimolar absorbancy of the phenylpyruvate-enol-borate complex at 300 mμ is 9.15 (37). One enzyme unit is the amount of enzyme transforming 1 μmole per minute of L-phenylalanine under these conditions. The activity is expressed as units per gram wet weight of mycelium.

ASSAY OF NADase ACTIVITY

NADase activity was assayed by a modification of the method of Kaplan, Colowick and Nason (38). The reaction mixture, containing 0.10 ml of appropriately diluted enzyme extract and 0.30 ml of 0.10M sodium phosphate buffer pH 7.2 was equilibrated for five minutes at 37°C. The reaction (cf. Appendix 1) was started by adding 0.10 ml of β-NAD (4 mg/ml) with rapid mixing. After $7\frac{1}{2}$ minutes the reaction was stopped by adding 3.0 ml of 1M potassium cyanide.

The absorbance at 325 mμ was measured on a Cary Model 15 spectrophotometer. The millimolar absorbancy of the NAD-CN complex is 6.30 (39). One enzyme unit is the amount 1 μmole of NAD per minute under these conditions. The activity is expressed in units per gram wet weight of mycelium.

THE INCORPORATION OF RADIOACTIVE AMINO ACIDS BY NEUROSPORA MYCELIA

The method used is that of Dr. Marguerite Fling of this laboratory (29). The mycelium, either water-washed or EDTA-treated, was suspended in buffer with the additions noted in each table. After about an hour, the C^{14}-amino acid was added to the buffer and the flasks were shaken for 15 minutes on a rotatory shaker at a very slow speed. They were then removed to an ice bath and 2.0 ml of 0.01M C^{12}-amino acid was added to each flask. The mycelial pads were harvested by suction on a Buchner funnel and rinsed four times with 10 ml of distilled water. The pad was removed and stored at -15°C in aluminum foil. The funnel and suction flask were rinsed twice with 10 ml of distilled water. All the buffer and water rinses were combined, the volume measured, and then stored frozen at -15°C until counted. Any CO_2 evolved during the experiment was not trapped.

EXTRACTION OF THE INCORPORATED AMINO ACIDS FROM THE MYCELIUM

The method for extraction of the amino acids from the mycelium was developed by Roberts et al. for Neurospora (40). The mycelium is extracted for 30 minutes at 4°C with ice-cold 5% trichloroacetic acid (TCA) in a centrifuge tube. The extract is removed and the mycelium extracted again for 10 minutes at 4°C with 5% TCA. These two extracts, containing the free amino acids of the mycelium, are combined as the cold TCA soluble fraction. Any residual TCA is wiped out of the tube. The mycelium is extracted next with 75% ethanol at 40-50°C for 30 minutes and the extracts removed. Then it is extracted with ether-75% ethanol (1: 1) for 30 minutes at 40-50°C, and the two extracts combined. In Neurospora this ethanol-soluble fraction contains mostly lipids. After drying for 15 minutes at 60°C, 5% TCA is added and the tubes are placed in a water bath. The water bath is then brought slowly to boiling to avoid bumping. The mycelia are held in the 5% TCA by glass rods to insure effective extraction. The pads are extracted for 75 minutes in the boiling water bath. The hot TCA-soluble extract contains mainly RNA and those amino acids attached to s-RNA. The pads are washed once with 75% ethanol and once with ether, and the washes are discarded. Then the pads are dried at 60°C for 30 minutes. The proteins in the mycelium are extracted overnight at

room temperature with 3% sodium hydroxide. The mycelium remaining is discarded.

PREPARATION OF SAMPLES FOR COUNTING AND COUNTING OF SAMPLES

An aliquot of each fraction was placed in a glass scintillation vial (Packard) and dried under a heat lamp. Then 0.20 ml Hyamine 10-x (Nuclear Chicago) was added to redissolve the residue and 15 ml of Liqui-fluor scintillation fluid (Nuclear Chicago) was added. The residue of the sodium hydroxide fraction was redissolved in 1.0 ml hyamine 10-x. The samples were counted, using the channel ratios method, in a Nuclear Chicago Series 720 liquid scintillation system. The amount of quenching was the same for all samples, so the data were not corrected for quenching.

RESULTS

I. STUDIES ON NADase

A. The Coinduction of NADase, Tyrosinase and L-Amino Acid Oxidase in 69-1113a

The enzymes tyrosinase and L-amino acid oxidase are induced together during starvation in phosphate buffer (13). When Fling and Horowitz (unpublished) found that NADase was present in induced cultures of wild-type 69-1113a, it was decided to study the induction of all three enzymes as a function of time.

Two-day-old cultures of 69-1113a were washed with water and induced in sodium phosphate buffer. At various times after the start of induction, duplicate cultures were harvested and the amount of each enzyme was measured. The amount of NADase in the induction buffer was also measured. Two separate experiments are shown in figures 1 and 2. As shown in figure 1, the time course of tyrosinase and L-amino acid oxidase were closely parallel, rising and falling together, in agreement with the findings of Horowitz (13). The time course of NADase was strikingly different. NADase appeared ten hours earlier than the other enzymes, rose to its maximum value earlier and then fluctuated about that maximum. In figure 2, another time course of tyrosinase and NADase induction is plotted. It is apparent that in this experiment tyrosinase and

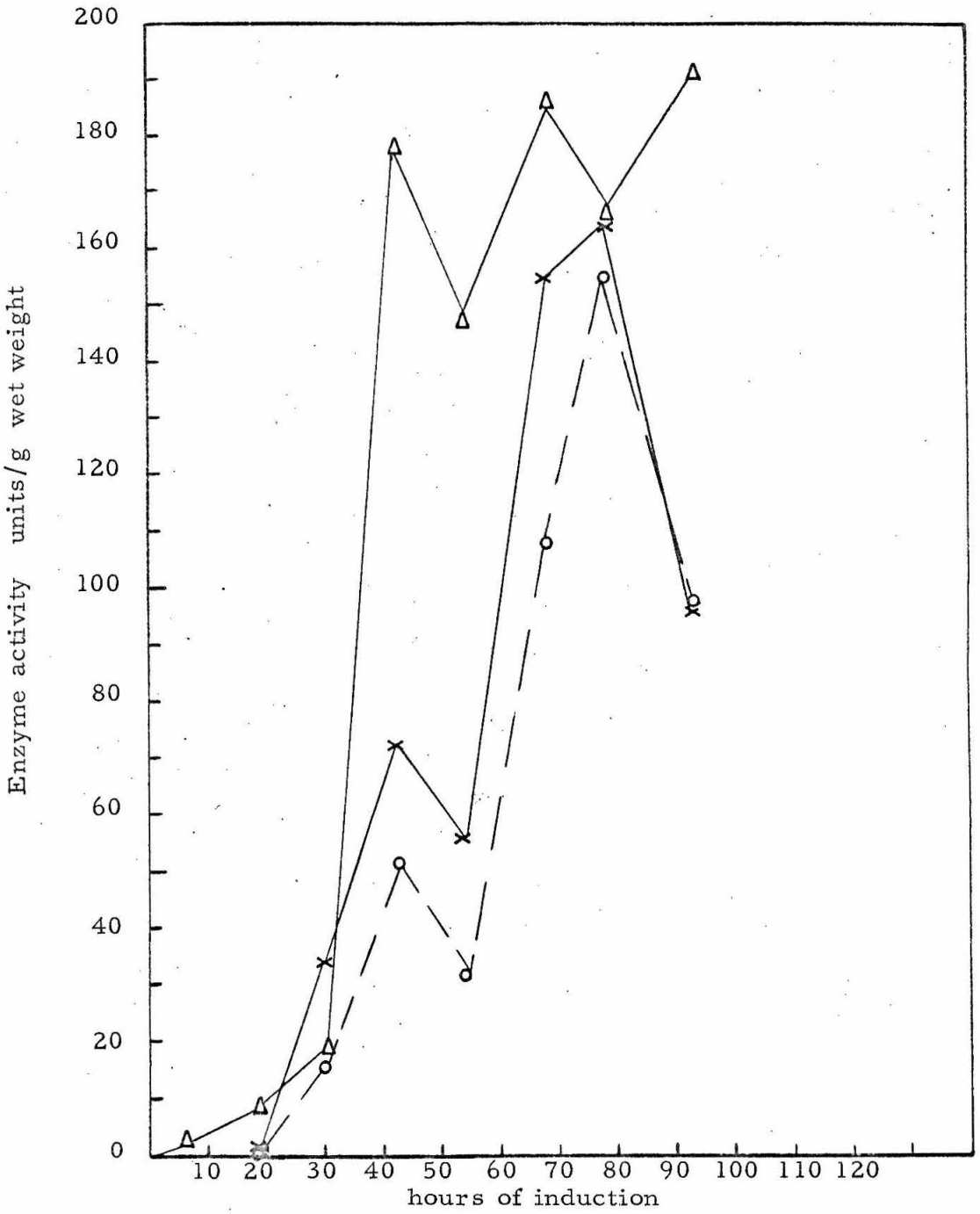

Figure 1. The time course of induction of tyrosinase, L-amino acid oxidase and NADase in 69-1113a. Two-day-old cultures of 69-1113a were washed with water and induced in .02M $NaPO_4$ buffer pH 6.0 until harvested, and assayed for each enzyme. X——X tyrosinase units/g; O---O L-amino acid oxidase units/g (40x); Δ——Δ NADase units/g (2x).

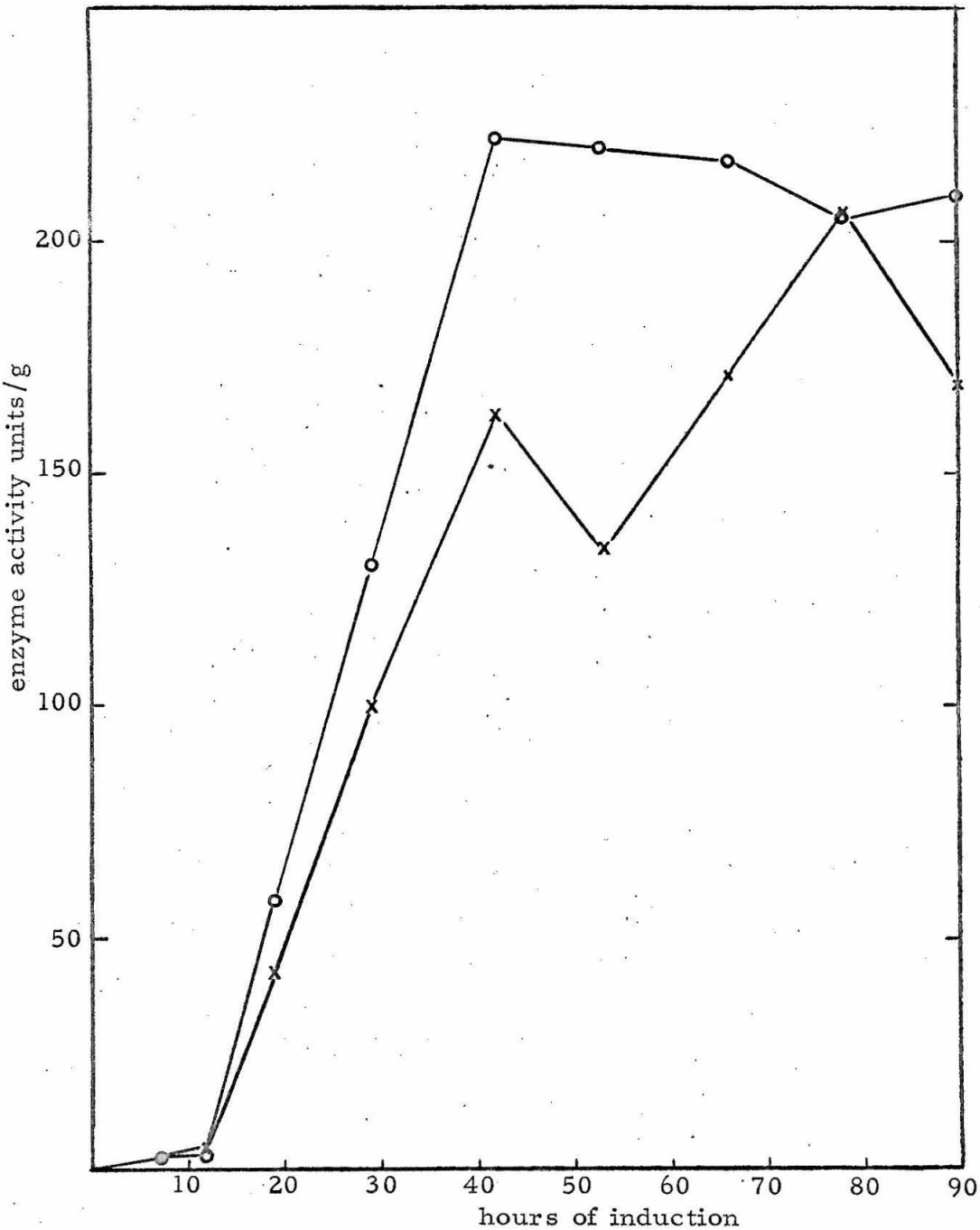

Figure 2. The time course of induction of tyrosinase and NADase in 69-1113a. Two-day-old cultures of 69-1113a were washed with water and induced in .02M $NaPO_4$ buffer pH 6.0 until harvested and assayed for each enzyme.
O——O tyrosinase X——X NADase (4X)

Table 2. The Ratio of Enzyme Activities During the Course of Induction of 69-1113a. (The data in the table are those used in drawing figures 1 and 2.)

Figure 1:

Hrs of induction	Ty'ase u/g	L-Ox'ase u/g	NADase u/g	Ratio of Activities: Ty'ase / Ox'ase	Ratio of Activities: Ty'ase / NADase
0	0	0	0	---	---
6	0	0	0.9	---	$\frac{0}{0.9}$
$18\frac{1}{2}$	1.1	0	4.4	$\frac{1.1}{0}$	0.25
30	34	0.38	9.4	89	3.6
$42\frac{1}{2}$	72	1.29	88.5	56	0.81
54	56	0.79	73.4	71	0.76
68	155	2.69	92.8	58	1.7
78	164	3.87	82.7	42	2.0
93	96	2.44	95.4	39	1.0

Figure 2:

Hrs of induction	Ty'ase u/g	NADase u/g	Ratio of Activities: Ty'ase / NADase
0	0	0	---
$7\frac{1}{2}$	3.2	0.6	5.3
12	3.4	1.4	2.4
19	58	10.8	5.4
29	130	24.8	5.2
42	222	40.4	5.5
53	220	33.3	6.6
66	213	42.7	5.0
78	205	53.7	3.8
90	215	48.2	4.5

NADase rose together in a parallel manner, but that after the completion of the rise phase, the curves diverge.

The difference in the time courses of tyrosinase and L-amino acid oxidase induction on the one hand and that of NADase on the other is made more apparent in table 2, where the ratios of the enzyme activities shown in figures 1 and 2 are compared. The ratio of tyrosinase to L-amino acid oxidase activity decreased two-fold during the course of experiment #1. Tyrosinase, like other phenol oxidases, is destroyed during catalysis (35). Since old cultures are brown, presumably due to melanin formation by tyrosinase, the relative increase in L-amino acid oxidase may be due to the destruction of tyrosinase by this mechanism. The ratio of tyrosinase to NADase in figure 1 contained no obvious pattern and varied five-fold between successive times in the course of the experiment. In figure 2 the ratio of tyrosinase to NADase was higher and less variable.

The induction of NADase need not parallel the induction of tyrosinase (see figure 1), though it may during the early stages of induction (see figure 2).

Zalokar and Cochrane (28) have shown that maximal production of NADase in the wild type Em 5297a occurred during conidiogenesis. They report concentrations of 17,000 units/mg protein, which would be about 200 Enzyme Commission units per gram wet

weight of mycelium. Thus the values of NADase, 100 units/g wet weight, found during phosphate induction, are high enough that if they were associated with conidia, large numbers of conidia should be present. But large numbers of conidia were not observed during induction in phosphate buffer. Further, the wild type 69-1113a is not a heavy producer of conidia. Therefore, conidiogenesis is probably not the cause of the production of NADase in this case. It is possible that some of the conditions connected with conidiation are duplicated in these cultures. In particular, the amount of oxygen reaching the hyphae is increased by the switch from 20 ml of growth medium to 5 ml of induction buffer, and such changes may be sufficient to induce NADase synthesis.

B. The De Novo Synthesis of NADase During Induction

If the NADase activity appearing during induction were due to de novo synthesis of the enzyme, then the amount of activity should be reduced by inhibitors of protein synthesis. Puromycin is thought to inhibit protein synthesis by attaching to the growing end of the polypeptide chain on the ribosome in place of an amino acyl s-RNA (41, 49). The growing polypeptide then falls off the ribosome unfinished (47, 48, 49). When puromycin was added to the induction buffer, it decreased the amount of tyrosinase and NADase induced (table 3). No inhibitor of any of these enzymes was found in these

extracts. Nason et al. (27) had previously concluded that the differences in activity they detected were due to different amounts of enzyme in different cultures, rather than the presence of activators or inhibitors in the different cultures. It has been proved that tyrosinase is made de novo during induction (31). It is consistent with the foregoing results that NADase is synthesized de novo during induction.

Table 3. The Inhibition of Induction by Puromycin.

Additions to the induction buffer	Ty'ase u/g	NADase u/g
None	105	24
Puromycin	67	3.4
% Inhibition	36	86

Two-day-old cultures of 69-1113a were washed with water and induced for two days in .02M $NaPO_4$ buffer pH 6.0 with and without the addition of 10^{-3}M puromycin·2HCl.

If the conclusion that these enzymes are synthesized during induction is correct, then one may ask when the messenger RNAs corresponding to them are made. Actinomycin D inhibits the DNA-directed synthesis of RNA (43, 44). By preventing the synthesis of messenger RNA, it inhibits the synthesis of enzymes in bacteria (45). Actinomycin D inhibits the growth of Neurospora (46), presumably by preventing RNA synthesis. As can be seen in table 4, actinomycin D

Table 4. The Inhibition of Induction by Actinomycin D.

Additions to the induction buffer	Ty'ase u/g	L-Ox'ase u/g	NADase u/g
None	242	5.9	41
Actinomycin D[1]	132	3.1	14
% Inhibition	46	48	66

Two-day-old cultures of 69-1113a were washed with water and induced in .02M $NaPO_4$ buffer pH 6.0 with the additions noted.

[1] 16 γ/ml

inhibited the induction of all three enzymes. This was known previously for tyrosinase and L-amino acid oxidase (42). Thus some RNA synthesis is necessary during induction. The simplest explanation is that the messenger RNA corresponding to the three enzymes is synthesized during induction. It is not excluded, however, that ribosomal or transfer RNA must be synthesized during induction.

C. Inhibition of NADase Induction by Amino Acids.

During the course of these studies it was noticed that cultures induced with DL-ethionine invariably had very low levels of NADase activity. To check whether this was a specific effect of DL-ethionine, cultures of 69-1113a were induced in phosphate buffer containing certain amino acids. (see table 5) While DL-ethionine was the most inhibitory , L-methionine and D-phenylalanine were also effective inhibitors. L-serine may

have been inhibitory.

Table 5. The Inhibition of NADase Induction by Amino Acids.

Additions to buffer	Tyrase units/g	NADase units/g	% Inhibition of NADase
none	105	22	0
	12	26	
DL-ethionine	172	0	100
	185	2.3	90
L-methionine	94	5.2	78
	108	3.6	86
D-phenylalanine	91	14.4	40
	140	3.3	86
L-serine	110	16	33
	132	27	-12

Two-day-old cultures of 69-1113a were washed with water, starved two days in .02M $NaPO_4$ buffer pH 6.0 with the additions noted. All amino acids were present at 1 mg/5 ml buffer.

One possible explanation of the results is that the amino acids were being made into an inhibitor of NADase activity. This was tested by mixing extracts of the cultures in table 5 with active NADase. (table 6) The extracts of cultures induced in the presence of either DL-ethionine or L-methionine do not contain an inhibitor of NADase, as seen in table 6.

Table 6. Mixing Experiments to Detect an NADase Inhibitor in the Extracts of Cultures Induced in the Presence of Amino acids.

	Additions to enzyme	NADase (arbitrary units)
Experiment #1	None	0.338
	Extract of cultures induced with L-methionine present	0.343
Experiment #2	None	0.322
	Extract of cultures induced with DL-ethionine present	0.337

1 part enzyme + 1 part extract incubated 30 @ 30°C, then diluted and assayed.

For induction conditions, see table 5.

Thus certain amino acids inhibit the induction of NADase in wild-type during starvation in phosphate buffer. Zalokar and Cochrane (28) found that inhibition of conidiation inhibited NADase synthesis. Although amino acids are often added to media to promote conidiation, it may be that they delay the start of conidiogenesis while increasing the yield. Thus the use of casamino acids instead of ammonium nitrate as the nitrogen source in liquid Vogel's medium N delayed the appearance of aerial hyphae from about four days to six days of growth in cultures of 69-1113a.

D. An Inhibitor of NADase in the Mutant Ty-1

Strains of Neurospora carrying a mutation in the regulatory gene ty-1 are female sterile and do not make tyrosinase or L-amino acid oxidase during starvation in phosphate buffer unless certain inducers are present in the buffer (18, 31, 13). The ty-1 locus is unlinked to the tyrosinase structural gene. It was of interest to determine whether NADase induction was also controlled by this regulatory gene. Therefore, two-day-old cultures of ty-1 were washed and induced in phosphate buffer containing D-phenylalanine. Tyrosinase was made but no NADase appeared (table 7).

Table 7. NADase Induction in Ty-1.

Additions to the induction buffer	Ty'ase u/g	NADase u/g
None[1]	1	0
D-phenylalanine[1]	46	0
Uninduced[2]	0.5	0

[1] Two-day-old cultures of ty-1 were washed with water and starved for two days in .02M $NaPO_4$ buffer pH 6.0 with and without D-phenylalanine at 0.2 mg/ml.

[2] Four-day-old growing cultures of ty-1 were harvested, extracted, and assayed.

The absence of NADase in these cultures is not due to a defect in its structural gene, since activity has been detected in some extracts prepared by Dr. Marguerite Fling in this laboratory.

All the extracts not containing NADase activity were tested for an inhibitor of NADase in mixing experiments (table 8).

Table 8. Inhibitors of NADase in Ty-1 Extracts.

	Additions to active enzyme		NADase ΔA_{325}	% Inhibition
Experiment #1	None		0.262	0
	Extract of growing ty-1	#1	0.000	100
		#2	0.045	82
	Extract of ty-1 starved in phosphate buffer	#1	0.080	66
		#2	0.000	100
Experiment #2	None		0.190	0
	Extract of ty-1 induced with D-phenylalanine		0.058	70

1 part enzyme + 4 parts of extract were incubated 30 minutes @ 30°C and stored at 4°C until assayed.

Same extracts as used in table 7.

All the extracts of ty-1 cultures contained an inhibitor of NADase. In an attempt to determine the nature of the inhibitor, the extracts were dialyzed overnight at 4°C against two changes of .10M sodium phosphate buffer pH 6.0. The mixing experiments were repeated using the dialyzed extracts (table 9).

Table 9. The NADase Inhibitor in Dialyzed Extracts of Ty-1.

	Additions to active enzyme	NADase ΔA_{325}	% inhibition
Experiment #1	None	0.190	0
	Extract of growing ty-1	0.010	95
	Extract of ty-1 starved in phosphate buffer	0.080	58
Experiment #2	None	0.248	0
	Extract of ty-1 induced with D-phenylalanine	0.262	0

The extracts used in table 8 were dialyzed overnight against two changes of .10M $NaPO_4$ buffer pH 6.0. Then one part enzyme and four parts of extract were mixed, incubated 45 minutes @ 30°C, and stored at 4°C until assayed.

The inhibitor in the extracts of cultures induced with D-phenylalanine was dialyzable, but no NADase was revealed by the removal of the inhibitor from the extract. The severe inhibition of induction by D-phenylalanine reported in the previous section probably explains this absence. The inhibitor in growing or phosphate induced cultures was not removed by dialysis. It was destroyed by heating the extract in a boiling water bath for 10 minutes. Since it was non-dialyzable and thermolabile, the inhibitor in growing cultures is probably a protein.

It should be noted that the strain of ty-1 used in these experiments is not isogenic with 69-1113a. Thus the inhibitor may not be due to ty-1 but to some other difference between the two strains. The presence of the inhibitor should be checked when ty-1 is introduced into more closely related lines. If it is still present, then studies in a heterocaryon with 69-1113a would be extremely interesting. The mutant ty-1 is recessive in its other characteristics to its wild-type allele. However, the production of a protein inhibitor of NADase would be expected to be a dominant character. Such experiments were not carried out due to lack of time.

E. Induction of the Three Enzymes in the Mutant Ty-2

A second regulatory gene is known in Neurospora which controls the inducibility of tyrosinase, the gene ty-2. Strains carrying the recessive mutant allele are female-sterile and fail to make tyrosinase during starvation in phosphate buffer unless an inducer is present. The ty-2 locus is unlinked to either the structural gene for tyrosinase or the other regulatory gene, ty-1 (18, 31).

Figure 3 shows the time course of induction of L-amino acid oxidase and tyrosinase in ty-2 when it is induced in phosphate buffer containing D-phenylalanine. L-amino acid oxidase was detected 6 hours earlier than tyrosinase. Tyrosinase was found 12 hours after the start of induction. Both enzymes increased to a maximum at 7.9 hours and then decreased in concentration.

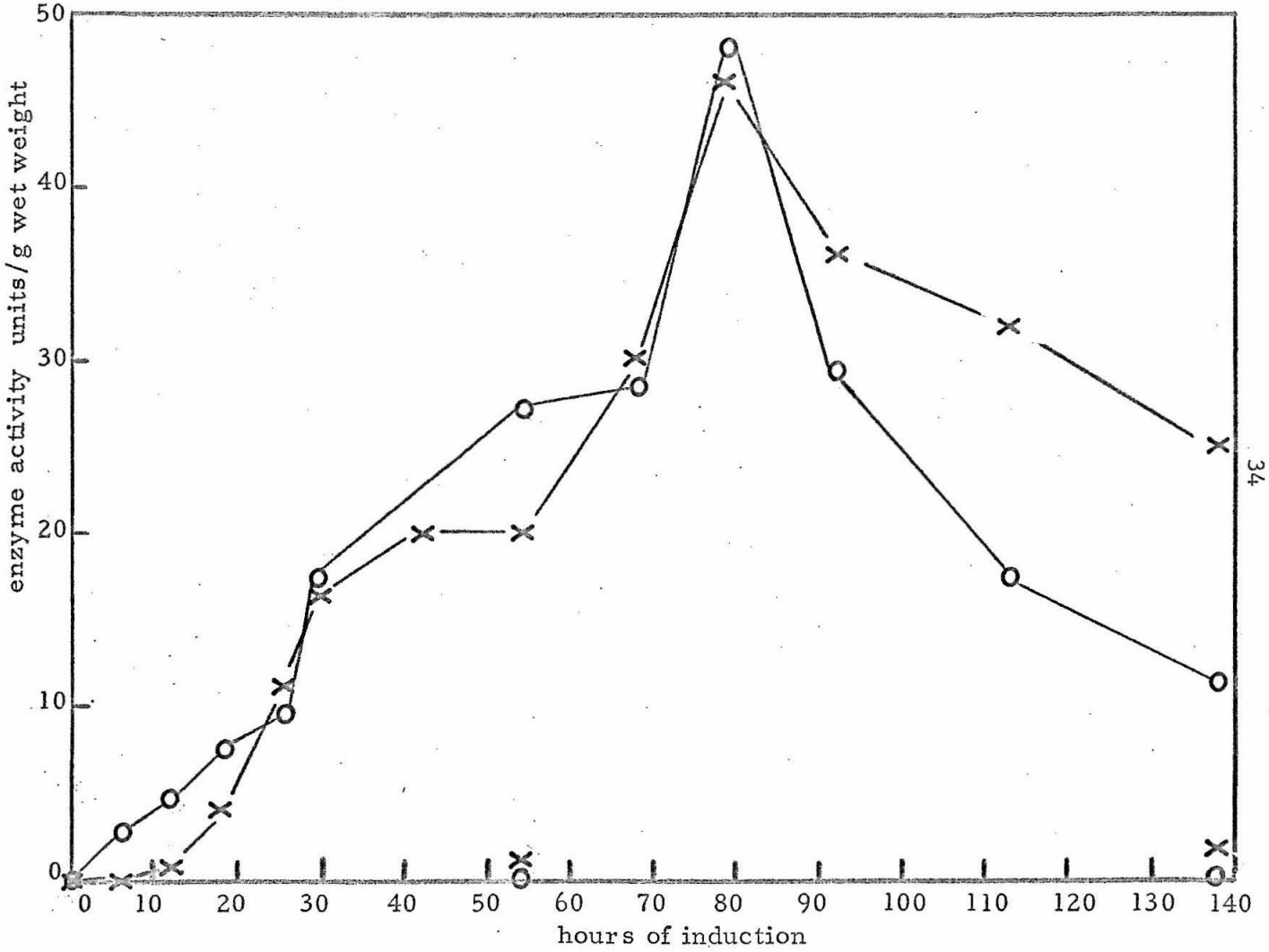

Figure 3. The time course of induction of tyrosinase and L-amino acid oxidase in ty-2. Two-day-old cultures of ty-2 were induced in .02M $NaPO_4$ buffer pH 6.0 with 0.2 mg/ml D-phenylalanine. X——X tyrosinase; O——O L-amino acid oxidase (40X) X,O controls no D-phenylalanine.

In the absence of D-phenylalanine, less than 1% of the maximum amount of tyrosinase was made and less than 5% of the maximum amount of L-amino acid oxidase was made. Thus ty-2 regulates L-amino acid oxidase synthesis in the same manner as it regulates tyrosinase synthesis.

NADase can be made by ty-2, but the time course and amount were not reproducible under the conditions used to induce tyrosinase and L-amino acid oxidase. In one experiment, the most enzyme was made in the absence of inducer, 5.5 u/g. The maximal amount ever detected was 10 u/g. These low levels were probably due to the inhibition of NADase induction by the D-phenylalanine used as inducer.

F. The Synthesis of NADase and L-Amino Acid Oxidase by Growing Neurospora

If the three enzymes, tyrosinase, L-amino acid oxidase, and NADase are controlled by a unitary system such as an operon or by a one-repressor-many-operons system, they should all be present or absent together in the cell. Two strains of Neurospora have been found in which this common control has broken down. In strain P110A, NADase and L-amino acid oxidase, but not tyrosinase, are made during growth. In the strain pe,fl;cot, only NADase is made during growth.

The strain P110A was given to us by Dr. Dow Woodward of Stanford University, who reported that it was constitutive for NADase. This was confirmed, and it was discovered that P110A also made L-amino acid oxidase but not tyrosinase while growing. It was decided to determine whether other strains in the laboratory made either of these enzymes during growth. Table 10 shows the results of three experiments in which various wild types and mutants of Neurospora were grown for two days on minimal medium N. All strains which made more than 0.5 u/g L-amino acid oxidase or 5 u/g NADase in either experiment #1 or #2 were tested at least two more times. Only P110A made large amounts of both enzymes consistently. A second strain, pe,fl;cot, made NADase consistently in large amounts. Several strains made some NADase or L-amino acid oxidase, but none made detectable amounts of tyrosinase. In particular, St 74A, the parent of P110A, did not make large amounts of L-amino acid oxidase or NADase. Mixing experiments with the extract of St 74A did not reveal the presence of inhibitors of either enzyme in it, indicating an absence of the enzymes from the extract. P110A was obtained by UV treatment of St 74A, and is probably due to a single mutational event. This event conferred on P110A the ability to synthesize during growth two enzymes which are not made by its parent during growth. The experiments do not indicate whether

Table 10. The Production of L-Amino Acid Oxidase and NADase by Growing Cultures of Neurospora.

Strain	Ty'ase u/g	L-Ox'ase u/g	NADase u/g
Experiment #1[1]			
1A	0	0.1	1
Abbott 4A	0	0.1	0.3
Abbott 12a	0	0.1	1.3
25a	0	0.15	0
Em 5297a	0	0.1	0
Scott A	0	0.1	0.1
pe,fl;cot[2]	0	0.3	12.4
P110A[2]	0	5.4	7.0
Experiment #2[1]			
1A	0	0	0.2
Abbott 4A	0	0.9	0.3
Abbott 12a	0	< 0.1	16
25a	0	< 0.1	0
Em 5297a	0	0.11	0.1
Scott A	0	0.19	2.0
pe,fl;cot[2]	0	0.15	55
P110A[2]	0	3.4	21
ty-1	0	0	0.6
ty-2	0	0	0
69-1113a	0	0	0.4
Experiment #3			
P110A[4]	0	1.74	12
St 74A	0	0.14	0
	0	0.12	
69-1113a induced[3]	141	1.55	43

[1] Cultures were grown on 20 ml of Vogel's minimal medium N in 125 ml Erlenmeyer flasks without shaking for two days and then harvested and the pad and medium assayed for the enzymes.

[2] Grown three days because of slow growth rate.

[3] Two-day-old cultures of 69-1113a were washed with water and induced for 68 hours in .02M sodium phosphate buffer.

[4] Grown four days.

the production of the two enzymes is a direct result of that event or whether one or both is synthesized because of some other effect of the mutation.

P110A differs from wild-type Neurospora in growing poorly on sucrose as the sole carbon source. P110A grows better when pyruvate is added as an additional carbon source. The strain pe,fl;cot also grows poorly on medium N with sucrose as carbon source. Since it is also constitutive for NADase, it would be interesting to know whether its growth is stimulated by pyruvate. The experiment has not yet been performed.

The results with P110A and pe,fl;cot suggested that the common control of tyrosinase, L-amino acid oxidase, and NADase had broken down in these strains. However, there were three other possible explanations: (1) the two strains might have defective structural genes for the missing enzymes; (2) an inhibitor of tyrosinase activity might be obscuring the presence of tyrosinase in these cultures; (3) inactive "protyrosinase" (51, 97, 98, 99, 100) might be present in these extracts. Mrs. Joyce Maxwell had previously found that pe,fl;cot could be induced to make both tyrosinase and L-amino acid oxidase. Her results and results of an induction of tyrosinase in P110A are shown in table 11. Tyrosinase was not made by P110A during 48 hours starvation in

phosphate buffer, but could be induced by ethionine or D-phenylalanine. Thus both strains possessed active structural genes for the enzymes which were not detected in growing cultures. As shown in table 12, the extracts of both strains did not contain any inhibitor of tyrosinase. To activate any "protyrosinase," the extracts were heated for 1-1/2 minutes at 59°C (51). As seen in table 12, no tyrosinase activity appeared in these extracts after heating. Therefore, the structural gene of tyrosinase and probably of L-amino acid oxidase was not active during the growth of the strain pe,fl;cot and that of tyrosinase was inactive in P110A. Thus the control of the three enzymes seems to be dissociated in these strains.

Table 11. The Induction of Tyrosinase and L-Amino Acid Oxidase in Strains P110A and pe,fl;cot.

Strain	Inducer	Ty'ase u/g	L-Ox'ase u/g
69-1113a	(starvation)	155	2.7
pe,fl;cot	DL-ethionine	28	2.4
P110A	(starvation)	0	---
P110A	DL-ethionine	108	---
P110A	D-phenylalanine	57	---

P110A grown three days. 69-1113a grown two days. P110A and 69-1113a induced in .02M sodium phosphate buffer pH 6.0 for two days. pe,fl;cot induced in 1X Vogel's basal salts (50). DL-Ethionine 0.2 mg/ml; D-Phe 1 mg/ml.

Table 12. The Absence of Tyrosinase Inhibitors and of Inactive Tyrosinase in Extracts of P110A and pe,fl;cot.

	Tyrosinase (arbitrary units)
Active enzyme	94
Enzyme + extract of uninduced P110A	96
Enzyme + extract of uninduced pe,fl;cot	94
Heat activated enzyme	126
Heat activated extract of pe,fl;cot	0
Heat activated extract of P110A	0

P110A and pe,fl;cot grown three days and harvested.

1 part enzyme + 2 parts extract of three-day-old cultures incubated 30 min @ 30°C and then assayed.

Heated 1-1/2 min @ 59°C and stored in ice until assayed.

Thayer and Horowitz (52) and Burton (53) reported that L-amino acid oxidase occurred in the growth medium of old cultures. It was not found in medium used to grow P110A.

G. The Identification of L-Amino Acid Oxidase in Extracts of P110A

The enzyme L-amino acid oxidase uses molecular oxygen as the hydrogen acceptor in its reaction with L-amino acids (53). The appearance of phenylpyruvic acid from L-phenylalanine could also

be due to a transaminase if a large amount of keto acid acceptor were present in the crude extract. To distinguish between these possibilities in the extracts of P110A, the assays were run under oxygen and air. That molecular oxygen was necessary for phenylpyruvic acid production is shown in table 13. Thayer and Horowitz (52)

Table 13. Dependence of Phenylpyruvic Acid Formation by Extracts of P110A on Oxygen.

P110A extract assayed under	Phenylpyruvate made mμmoles	O_2/air
O_2	163	3.2
air	51	

Extracts of P110A were assayed as in Materials and Methods except that one assay was performed under oxygen and the other under air.

reported a 3- to 6-fold increase in activity when the assay was performed under oxygen rather than air. Burton found a 3.4-fold increase (53). Thus the 3.2-fold increase measured for extracts of P110A proves the enzyme being assayed is authentic L-amino acid oxidase.

H. The Synthesis of NADase by P110A in the Presence of Tween 80.

Zalokar and Cochrane reported that when conidiation

was inhibited by growing Em 5297a at 35°C in the presence of Tween 80 (polyoxyethylene sorbitan monooleate Mefford), the synthesis of NADase was also inhibited (28). No obvious conidiation occurs during five days growth of P110A on Vogel's medium N, although by five days the pads are highly pigmented due to carotene formation. Since P110A does make NADase while growing at 35°C, it was decided to see how the conditions used by Zalokar and Cochrane (28) affected the production of NADase by P110A. Therefore, P110A was inoculated into Vogel's medium N containing 0.8% Tween 80 w/v, and grown for two or three days at 35°C. Controls were grown at 35°C in the absence of Tween 80. The results shown in Table 14 suggest that Tween 80 actually stimulated the synthesis of NADase in P110A. The extracts of both control cultures and Tween

Table 14. The Effect of Tween 80 on the Synthesis of NADase by P110A.

Medium	Age of culture (days)	Wet wt. g	Pad	NADase u/g Buffer	Total
minimal[1]	2	0.26	0.95	21.0	22.0
		0.21	0.39	17.1	17.4
+ Tween 80[2]	2	0.23	0.98	35.6	36.6
		0.21	1.3	43.2	44.5
minimal[1]	3	0.42	0	24.7	24.7
		0.36	17.5[3]	11.2	28.7
Tween 80[2]	3	0.33	0	45.1	45.1
		0.38	0	37.5	37.5

(footnotes to table 14):

[1] Vogel's minimal medium N
[2] Medium N + 0.8% Tween 80
[3] Extensive conidiation

80 cultures were checked for inhibitors or stimulators of NADase synthesis. As seen in table 15, none were present in the extracts. Preliminary results with L-amino acid oxidase showed no activity in either the extracts or the medium when Tween 80 was present in the medium; however, no check has yet been made for the presence of an inhibitor in these cultures.

Table 15. Absence of Inhibitors of NADase in Extracts of Cultures of P110A Grown on Medium Containing Tween 80

Addition to active enzyme	NADase activity (arbitrary units) observed	expected
None	154	160
Extract of 2-day-old culture grown on Vogel's	190	195
Extract of 3-day-old culture grown on Vogel's + Tween 80	178	170

1 part active enzyme + 4 parts extract or buffer heated 30 minutes @ 30°C and stored at 4°C until assayed.

Thus it would appear that Tween 80 does not inhibit the synthesis of NADase in the constitutive strain P110A. Time has not permitted experiments on the effect of Tween 80 on the constitutive synthesis of NADase by pe,fl;cot.

I. The Inhibition of the Synthesis of NADase and L-Amino Acid Oxidase in P110A by Complete Medium

The amount of NADase or L-amino acid oxidase P110A made during growth was affected by the medium in which it was grown. Table 16 shows the amounts of NADase and L-amino acid oxidase made by P110A when grown on liquid complete medium for different times and on Vogel's minimal medium N.

Table 16. The Effect of Growth Medium on NADase and L-Amino Acid Oxidase Synthesis by P110A.

Growth medium	Age (days)	Wet weight	NADase u/g	L-Ox'ase u/g
Complete[1]	1	0.05	2	---
	2	0.22	0.8	0.20
	2	0.34	<0.1	0.26
	2	0.29	<0.1	0.13
	2	0.32	<0.1	---
	2	0.11	1.2	0.33
Minimal Medium N	3	0.11	21	3.4
	4	0.14	7.4	5.4
	2*	0.12	6.9	2.6
	2*	0.125	7.1	2.7
	4*	0.44	20	1.4
	4*	0.36	7.5	0.8

* Grown at 35°C

[1] Vogel's minimal medium N + 1.5% malt extract, 0.5% yeast extract, 0.025% casamino acids + 2% glycerol.

Some component(s) of the complete medium suppressed the synthesis of both enzymes by P110A, but time did not permit its identification. The effect was not due simply to the greater amount of growth in complete medium. The mycelial pads grown four days at 35°C on minimal weighed more than any of the pads grown in complete medium and contained large amounts of both enzymes. The chronological age of the pads was not important, as shown by the large amounts of the enzymes in cultures grown two days at 35°C in minimal medium. It may be that the faster rate of growth of cultures on complete medium is an important factor. This repression of constitutive production in P110A by complete medium may be analogous to catabolite repression of constitutive production in the lactose operon of E. coli (54, 55, 56).

P110A grown on complete medium could be induced to make more of both enzymes (table 17). Thus growing P110A on complete medium suppressed the constitutive production of NADase and L-amino acid oxidase. In such circumstances the enzymes could be induced by starvation in phosphate buffer in the presence of an inducer, although oxidase production was less responsive. This situation may be analogous to the dependence of tyrosinase production by wild type on the composition of the growth medium (18, 31). When wild types were grown on normal Vogel's medium N, no tyrosinase

was made by growing cultures unless an inducer was added to the medium. However, large amounts of tyrosinase were made when wild type was grown on media which limited growth.

Table 17. The Induction of NADase and L-Amino Acid Oxidase in P110A Grown on Complete Medium.

Additions to induction buffer	NADase u/g	L-Ox'ase u/g
Experiment #1		
Not induced	0	0.13
None	0.6	0.23
D-phenylalanine	6	0.30
Experiment #2		
Not induced	0.5	0.23
None	1.1	0.30
D-phenylalanine	14	0.52

P110A grown two days on liquid complete medium and induced two days in .02M $NaPO_4$ buffer pH 6.0
D-phenylalanine 0.2 mg/ml

II. STUDIES WITH EDTA

A. The Inhibition of Induction by EDTA

Neurospora tyrosinase contains one atom of cuprous copper per molecule (35). Although the enzyme contains no cysteine residues, the copper is tightly bound. In contrast to mushroom (58), animal (59), and potato (60) phenol oxidases, it has not been possible to remove the copper from the enzyme in a reversible manner. On the other hand, apo-tyrosinase has been detected in mushrooms (57). It was desired to obtain the apo-enzyme from Neurospora in order to study it and to label the active site of the enzyme with radioactive copper. The chelating agent EDTA forms a very firm complex with cupric copper at pH 6.0 (78). Since cupric ion is the stable form of copper in solution at room temperature, it was expected that treating the mycelium with EDTA would remove almost all of the copper in the mycelium. In the absence of copper, it was hoped that Neurospora would make the apo-enzyme for us and that we would be able to isolate and study it. In a later section it is reported that EDTA does not remove a large fraction of the copper in the mycelium so that the expectation voiced above is probably not realizable. Nevertheless, the experiments with EDTA have revealed some interesting points connected with the induction process.

Two-day-old cultures of Neurospora were treated with EDTA and induced in phosphate buffer. As seen in table 18 , induction of tyrosinase was inhibited by the EDTA treatment. In addition, L-amino acid oxidase and NADase induction were inhibited. In all cases, less than 5% of normal amounts of any of the enzymes appeared.

Table 18. Inhibition of Induction by EDTA Treatment.

	Ty'ase u/g	L-Ox'ase u/g	NADase u/g
Control	156	2.75	20
EDTA washed	< 1	0.07	0

Two-day-old cultures were washed with EDTA and harvested after two days in 5 ml .02M sodium phosphate buffer pH 6.0. Controls were washed with water and induced in buffer for two days.

B. The Restoration of Induction in EDTA-Treated Cultures.

As soon as the inhibition was discovered, experiments were undertaken to see whether the induction of tyrosinase and later the other enzymes could be restored by adding chemicals to the induction buffer. It was found that induction of tyrosinase and L-amino acid oxidase was restored to control levels by adding calcium, copper, and L-amino acids to the induction medium as shown in table 19. Experiments with other ions are reported in sections II E.,F., G., & I The effects of calcium, copper, and amino acids in restoring

Table 19 . Restoration of Tyrosinase and L-Amino Acid Oxidase Induction After EDTA.

Additions to buffer	Ty'ase u/g	L-Ox'ase u/g
Water-washed control	156	2.7
EDTA-treated:		
None	0.1	0.06
L-amino acids	0.2	0.30
Calcium	6	0.18
Copper	14	0.69
Calcium + copper	33	1.4
Calcium + copper + L-amino acids	104	2.8
Calcium + L-amino acids	5.2	0.13
Copper + L-amino acids	26	1.0

Two-day-old cultures of 69-1113a were treated with EDTA and induced for two days in .02M $NaPO_4$ buffer with the additions noted. $CaCl_2$ 5×10^{-5}M; $CuSO_4$ 5×10^{-4}M; L-amino acids 2×10^{-3}M each.

induction were synergistic. It was interesting that copper should have such a great effect on the restoration of L-amino acid oxidase synthesis, since that enzyme is an FAD enzyme rather than a copper enzyme like tyrosinase. Experiments on the ability of nickel to replace copper in the restoration probably can explain this result (cf. p. 60).

No combination of salts and amino acids has yet been found which gives complete restoration of NADase induction as shown in table 20.

Table 20 . The Restoration of NADase Induction After EDTA Treatment.

Additions to buffer	Ty'ase u/g	NADase u/g
Water-washed control	49	38
EDTA-treated:		
None	0.8	0
Copper	30	2.9
Copper + L-amino acids	37	0.8
Calcium + copper	54	10
Calcium + L-amino acids + copper	152	2.2
Calcium	0.4	0
Calcium + L-amino acids	1.6	0.8
L-amino acids	0.4	0
Vogel's basal salts[1]	37	17

Two-day-old cultures of 69-1113a were treated with EDTA and induced for two days in .02M $NaPO_4$ buffer pH 6.0 with the additions noted.

$CaCl_2$ 5×10^{-5}M; $CuSO_4$ 5×10^{-4}M; L-amino acids 2×10^{-3}M each.

[1] Mineral salts of Vogel's medium N

It is apparent that amino acids were strongly inhibitory to the restoration of NADase induction at this concentration. This is to be expected since a solution of 2×10^{-3} M L-methionine contains about 0.3 mg/ml L-methionine, and it was shown previously that L-methionine strongly inhibits NADase synthesis at a three-fold higher concentration (cf. p. 27).

The effects of the ions and amino acids which restored induction in EDTA-treated cultures on tyrosinase induction in control cultures are shown in table 21. Two-day-old cultures of 69-1113a were washed with water and induced in buffer to which calcium, copper, and amino acids were added. The addition of amino acids to the buffer had very little or no effect on the amount of tyrosinase made. Calcium was inhibitory at this concentration. Copper also inhibited, while a combination of calcium and copper fell between the amount of inhibition caused by the individual ions as if the two ions were acting on the same site in a competitive manner. No assays of L-amino acid oxidase or NADase were performed on these cultures.

C. Apo-tyrosinase and the EDTA Treatment

The following experiments were devised to detect apo-tyrosinase in EDTA-treated cultures. If apo-tyrosinase were being made in EDTA-treated cultures, then the addition of calcium and

Table 21 . The Effects of Calcium, Copper, and L-Amino Acids on Tyrosinase Induction in Water-Washed Cultures.

Additions to the induction buffer	Ty'ase u/g	% inhibition
None	128	---
L-amino acids	106	17
Calcium	4.9	96
Calcium + amino acids	6.2	95
Copper	97	24
Copper + amino acids	96	24
Calcium + copper	25	80
Calcium + copper + amino acids	30	76

Two-day-old cultures of 69-1113a were washed with water and placed in 5 ml of .02M $NaPO_4$ buffer pH 6.0 containing the additions shown.
L-amino acids 2×10^{-3}M each; $CaCl_2$ 5×10^{-5}M; $CuSO_4$ 5×10^{-4}M
Cultures were harvested after two days induction.

copper near the end of induction should lead to the immediate appearance of large amounts of tyrosinase. Figure 4 shows that this was not the result in an experiment in which calcium and copper were added to phosphate buffer 0, 8, 18, and 30 hours after the cultures were washed with EDTA. This suggests that no apo-tyrosinase which could be activated by the addition of copper and calcium was present in the cultures.

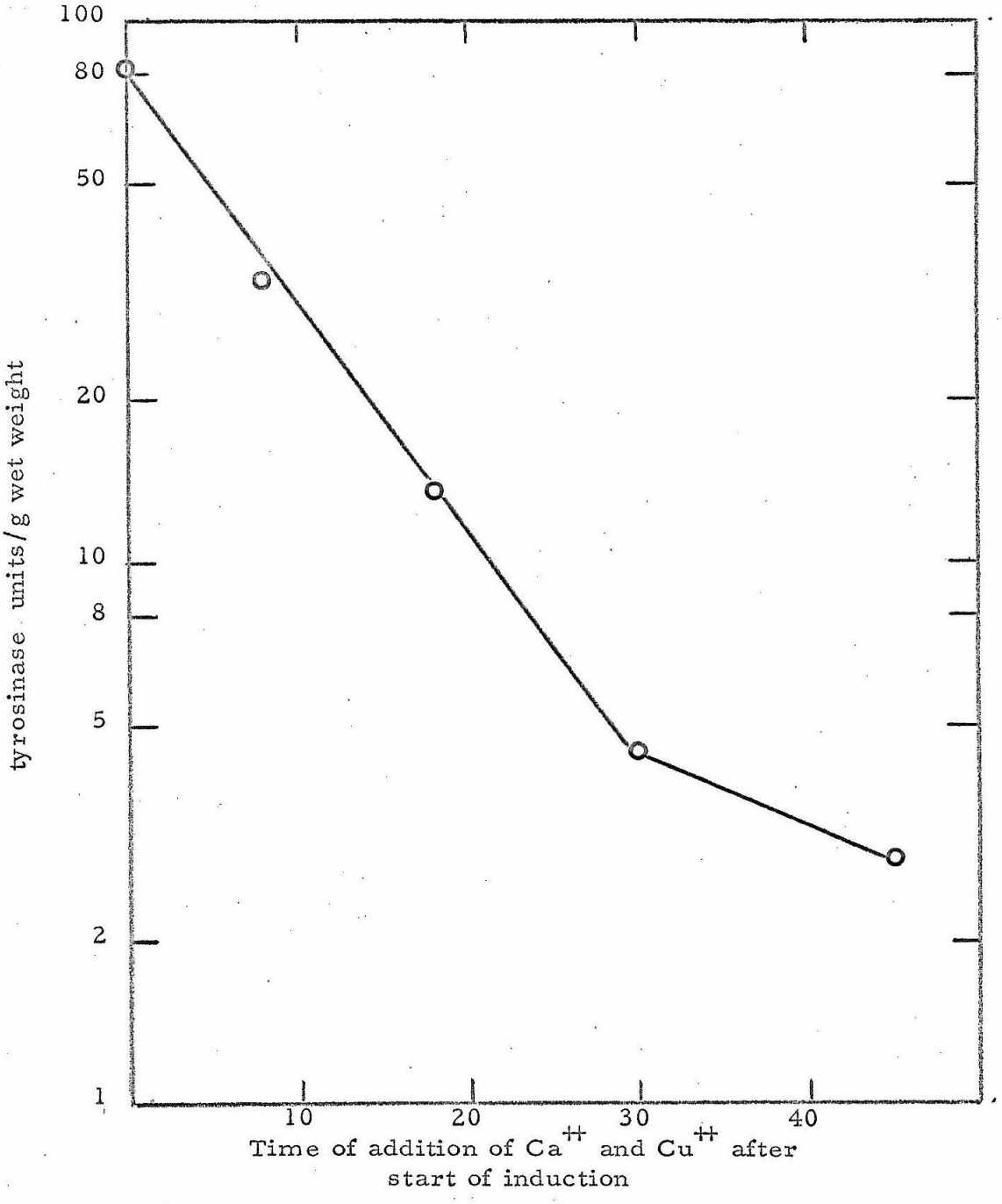

Figure 4. The amount of tyrosinase made upon addition of calcium and copper to EDTA-treated cultures as a function of the time of addition. Two-day-old cultures of 120-1 T^{sing} were washed twice with buffer, once with EDTA, and rinsed once with buffer before being placed in .02M $NaPO_4$ pH 6.0. At 0, 9, 18, 30, and 45 hours, after being placed in buffer, 5×10^{-5}M $CaCl_2$ and 5×10^{-4}M $CuSO_4$ were added to the buffer. All cultures were harvested 45 hours after the start of induction.

Experiments to detect apo-tyrosinase were also carried out on extracts of cultures treated with EDTA. Extracts were prepared and treated with cupric and cuprous copper, both in the presence and absence of 3M urea at both 4°C and room temperature. Since tyrosinase loses its copper irreversibly at pH's below 4.5, some experiments were performed at about pH 5, where it might be expected that tyrosinase would be much more stable than apo-tyrosinase. No procedure resulted in the appearance of tyrosinase activity in the extracts.

Thus no evidence of the presence of apo-tyrosinase in EDTA-treated cultures exists. Some evidence which suggests it should not be found in such cultures will be found in later sections of this thesis and commented on at that time. One fact that suggests that EDTA acts in a more general manner than by removing copper is the inhibition of the induction of L-amino acid oxidase, an FAD enzyme, (53) and NADase, an enzyme with no known prosthetic group (38).

Figure 4 is intriguing because of the linear relationship between the logarithm of tyrosinase content and time. Examination of the time courses of induction reveals that the amount of tyrosinase in the early synthetic phase of induction often increases exponentially. These kinetics of induction imply that the amount of enzyme which can be made depends on the amount of enzyme already present, i.e.

that induction is autocatalytic. This suggestion will be developed further in the discussion.

D. The Absence of Inhibitors of Any of the Enzymes in Extracts of EDTA-Treated Cultures

If EDTA treatment caused the production of an enzyme inhibitor, then a concomitant production of that enzyme might not be detected. This possibility was eliminated for each enzyme by mixing active extracts with extracts from EDTA-treated cultures lacking activity, as shown in table 22.

Table 22. The Lack of Inhibitors in Extracts of EDTA-Treated Cultures

Enzyme	Additions to enzyme	Activity (arbitrary units)
Tyrosinase	None	32
	Extract[1,2]	33
NADase	None	0.354
	Extract[1,3]	0.348
L-amino acid Oxidase	None	0.253
	Extract[1,2]	0.264

[1] Extracts of cultures of 69-1113a treated with EDTA and induced for two days in .02M $NaPO_4$ buffer pH 6.0.

[2] 1 part enzyme + 1 part extract incubated 30 minutes @ 30°C and then stored at 4°C until assayed.

[3] 1 part enzyme and 9 parts extract incubated 30 minutes @ 30°C and then stored at 4°C until assayed.

The extracts from EDTA-treated cultures did not contain any detectable inhibitor of tyrosinase, NADase, or L-amino acid oxidase.

Therefore, EDTA prevents the appearance of these three enzymes during starvation in phosphate buffer.

E. The Optimal Concentrations of Calcium and Copper Required to Restore Maximal Induction of Tyrosinase and L-Amino Acid Oxidase After EDTA Treatment

As reported above, preliminary experiments indicated that some restoration of induction occurred when calcium and copper were present in the phosphate buffer after EDTA treatment. At the same time, experiments by Miss Helen Macleod (87) on another system suggested that fairly high concentrations of an amino acid mixture might stimulate recovery from EDTA, which they did. (cf. table 19) Therefore, it was decided to determine what concentrations of calcium and copper in the presence of the amino acid mixture restored tyrosinase and L-amino acid oxidase induction in EDTA-treated cultures.

Two-day-old cultures of 69-1113a were treated with EDTA and suspended in 0.02M sodium phosphate buffer pH 6.0 containing 2×10^{-3}M L-amino acids (each). Varying concentrations of calcium and copper were added to the buffer. After two days the cultures were harvested and the amount of tyrosinase and L-amino acid

Table 23 . The Effects of Different Amounts of Calcium and Copper on the Restoration of Induction of Tyrosinase and L-Amino Acid Oxidase After EDTA Treatment.

Amount added to buffer M		Ty'ase u/g	L-Ox'ase u/g
$CaCl_2$ M	$CuSO_4$ M		
Control		107	4.8
10^{-3}	0	48	0.66
10^{-3}	10^{-2}	0	0
10^{-3}	10^{-3}	14	0.13
10^{-3}	10^{-4}	46	0.62
10^{-3}	10^{-5}	58	0.49
10^{-4}	0	20	1.8
10^{-4}	10^{-2}	0	0
10^{-4}	10^{-3}	158	6.4
10^{-4}	10^{-4}	92	4.7
10^{-4}	10^{-5}	34	2.3
10^{-5}	0	16	0.92
10^{-5}	10^{-2}	0	0
10^{-5}	10^{-3}	124	6.7
10^{-5}	10^{-4}	22	1.4
10^{-5}	10^{-5}	6.6	0.58
10^{-6}	0	2.5	0.47
10^{-6}	10^{-2}	0	0
10^{-6}	10^{-3}	60	4.4
10^{-6}	10^{-4}	1.8	0.49
10^{-6}	10^{-5}	0.7	0.61
None		0.5	0.13

(footnote on next page)

Footnote to Table 23

Two-day-old cultures of 69-1113a were treated with EDTA and induced for two days in .02M $NaPO_4$ buffer pH 6.0 containing a mixture of L-amino acids at 2×10^{-3}M each and the additions noted. Controls were washed with water and induced in .02M $NaPO_4$ buffer pH 6.0 for two days.

Table 24. Optimal Concentrations of Ca^{++} and Cu^{++} for Restoration of Tyrosinase and Oxidase Synthesis.

Amount added to buffer (molar)		Ty'ase u/g	L-Ox'ase u/g
$CaCl_2$	$CuSO_4$		
$3x10^{-4}$	0	136	1.5
$3x10^{-4}$	10^{-3}	72	1.2
$3x10^{-4}$	$3x10^{-4}$	114	1.1
$3x10^{-4}$	10^{-4}	181	3.0
10^{-4}	0	102	6.8
10^{-4}	10^{-3}	216	6.9
10^{-4}	$3x10^{-4}$	258	10.6
10^{-4}	10^{-4}	182	7.7
None		0.6	0.17

See table 23 for experimental procedures.

oxidase in each determined. As can be seen in table 23, the optimal concentration of calcium was between 10^{-5} and 10^{-4} gm ion/liter in the presence of 10^{-3} gm ions/liter copper. Table 24 shows the results of a similar experiment in which the concentrations were varied over a smaller range. Other experiments have confirmed the finding that about 10^{-4} gm ions/liter calcium plus about 3×10^{-4} gm ions/liter copper in the presence of 2×10^{-3} M L-amino acids (each) are the optimal conditions for restoring the induction of tyrosinase and L-amino acid oxidase.

Higher calcium concentrations were much more inhibitory to L-amino acid oxidase restoration than to tyrosinase restoration. An interesting feature of the EDTA-treated cultures is that when restored with copper, calcium, and amino acids, they often produce more enzyme than the controls. This is not due to the greater number of times they were washed, which suggests that EDTA has removed some inhibitory cation.

F. The Role of Copper in the Restoration of Tyrosinase Induction After EDTA Treatment

Copper is complexed very strongly by EDTA at pH 6.0 (log K' = 14.14) (78)[1] and helps restore the induction of tyrosinase

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[1] The formation constant of the metal EDTA complex is:

$$K_{MY} = \frac{(MY^{(n-4)+})}{(M^{n+})(Y^{-4})}$$

where (MY) is the concentration of the complex (M^{n+}) is the concentration of free metal ion and (Y^{-4}) is the concentration of the free tetra anion. The conditional formation constant is:

$$K'_{MY} = \frac{(MY)}{(M^{n+})(C_Y)}$$

where C_Y = the total concentration of free EDTA. $C_Y = (Y^{-4})/\alpha_4$, where α_4 is the fraction of free EDTA present as the Y^{-4} anion, and is a function of pH.

Thus,

$$\log K'_{MY} = \log K_{MY} + \log \alpha_4$$

is a measure of the strength of the complex at any given pH.

after EDTA-treatment. However, no change in the copper content of the ash of Neurospora was detected due to EDTA-treatment (cf. p. 74). If no significant amount of copper is removed by EDTA, then why does added copper help restore tyrosinase induction following EDTA treatment? The possibility that the inhibition caused by EDTA is due to EDTA remaining in the mycelium and binding some vital cation suggests itself. Since copper is bound more strongly by EDTA than most other cations, it might act by complexing all the EDTA, thereby freeing the essential cation(s). If this is how copper acts, then nickel (II), which is bound as strongly as copper (II) by EDTA at pH 6 ($\log K' = 13.96$) (78), would be expected to have the same effect on restoring tyrosinase induction as copper. Further, since nickel has no known biological function and is chemically different from copper, it would not be expected to replace copper in any enzymatic function.

To test this hypothesis, two sets of cultures of 69-1113a were treated with EDTA and placed in phosphate buffer containing amino acids and a low concentration of calcium (3×10^{-5} gm ions/liter). To one set copper sulfate was added in concentrations from none to 10^{-3}M, while nickel chloride in the same concentrations was added to the other set. After two days of induction, the two sets were harvested and their tyrosinase contents determined.

It can be seen in table 25 that nickel and copper had the same large stimulatory effect on the amount of tyrosinase made by EDTA-treated cultures. Thus it is probable that some EDTA remains in the treated mycelium after rinsing with water to remove it, and that copper and nickel assist the restoration of tyrosinase by binding that EDTA.

Table 25 . The Effects of Copper and Nickel on the Restoration of Induction After EDTA Treatment.

Molarity of $CuSO_4$ or $NiCl_2$	Tyrosinase Activity u/g	
	$CuSO_4$	$NiCl_2$
0	27	
10^{-6}	17	21
10^{-5}	18	21
10^{-4}	184	180
10^{-3}	142	144
Water-washed control	84	
EDTA-treated control	1.0	

Two-day-old cultures of 69-1113a were treated with EDTA and induced for two days in .02M $NaPO_4$ buffer pH 6.0 containing 3×10^{-5}M $CaCl_2$ and L-amino acids 2×10^{-3}M (each) and either $CuSO_4$ or $NiCl_2$ as noted.

The retained EDTA must be bound to a cation which can be displaced or replaced by calcium, since when the concentration of calcium is high enough, no added copper is necessary to obtain maximal synthesis of tyrosinase. The most reasonable candidate for this cation is calcium itself, since the fact that calcium must be added to the mycelium even in the presence of copper in order to get full restoration implies that it is not present in adequate amounts in EDTA-treated mycelium.

G. Other Cations.

Other ions were tested less critically than nickel and copper on EDTA-treated cultures. Although some stimulation of restoration above that caused by calcium alone was observed, they were all less effective than copper. These ions were Mn^{++}, Zn^{++}, and $MoO_4^{=}$. Both Ag^{+} and Hg^{++} completely inhibited restoration at 10^{-6} gm ions/liter.

During the earliest work with EDTA-treated cultures, it was assumed that copper would be essential and therefore tests centered on replacing calcium by other ions. The effect of magnesium was studied intensively and is reviewed in the next section. Among other cations tried, only strontium appeared capable of replacing calcium. Cations which did not replace calcium under the conditions and at the concentrations tried, were K^{+}, Ni^{++}, Zn^{++}, Fe^{+++}, Mn^{++},

and Co^{++}. The ability of strontium to replace calcium in biological systems is not unusual. The best known example is the ability of strontium to be deposited in bones in place of calcium (91).

H. Magnesium and the Restoration of Tyrosinase Induction After EDTA-Treatment.

One common use of EDTA is to remove magnesium from biochemical systems requiring it. For example, magnesium stabilizes ribosomes *in vitro*, and EDTA is used routinely to break ribosomes down into their sub-units (92, 93). Like calcium, magnesium did not appear to be removed from the mycelium by EDTA according to the spectrographic analysis (cf. p. 74). However, since calcium and magnesium are similar chemically, it was suggested that magnesium might have the same effect as calcium on the restoration of tyrosinase induction after EDTA treatment. Two duplicate sets of cultures were treated with EDTA and identical additions were made to the phosphate buffer except that calcium was added to one set and magnesium to the other. Table 26 shows that magnesium either had no effect or decreased the amount tyrosinase induction restored.

Table 26 . Magnesium and the Restoration of Induction After EDTA Treatment.

Additions to buffer	Molarity $MgSO_4$	Ty'ase u/g
Water-washed control	0	49
EDTA-treated: None	0	1
or calcium	10^{-5}	0
or L-amino acids	10^{-4}	0
	0	30
Copper	10^{-5}	18
	10^{-4}	13
	0	37
Copper, L-amino acids	10^{-5}	40
	10^{-4}	41
	0	54
Calcium, copper	10^{-5}	33
	10^{-4}	29
	0	152
Calcium, copper, L-amino acids	10^{-5}	93
	10^{-4}	116
	0	1.6
Calcium, L-amino acids	10^{-5}	0
	10^{-4}	4

Two-day-old cultures of 69-1113a were treated with EDTA and induced in .02M $NaPO_4$ buffer pH 6.0 containing the additions noted. $CaCl_2$ 5×10^{-5}M; $CuSO_4$ 5×10^{-5}M; L-amino acids 2×10^{-3}M (each).

When the data are compared, as in table 27 , a further point appears.

Table 27 . Effects of Magnesium and Calcium on the Restoration of Induction After EDTA Treatment.

Present in induction buffer	Additional cation	Ty'ase u/g	% change
Copper	None	30	---
	Calcium	54	+80
	Magnesium	18	-40
Copper, L.-amino acids	None	37	---
	Calcium	152	+310
	Magnesium	41	+11

Data from table 26

The addition of calcium to buffer containing copper or copper and amino acids caused a large increase in the amount of tyrosinase made, whereas the addition of magnesium caused either a decrease or no change. One explanation of this result is that the EDTA does not remove ions from the inside of the mycelium where magnesium is important, but only from the outside and membrane where calcium is important. A second explanation is that magnesium may be in such great excess in mycelia grown on Vogel's medium N that EDTA cannot remove enough of it to have any effect. The growth curve in

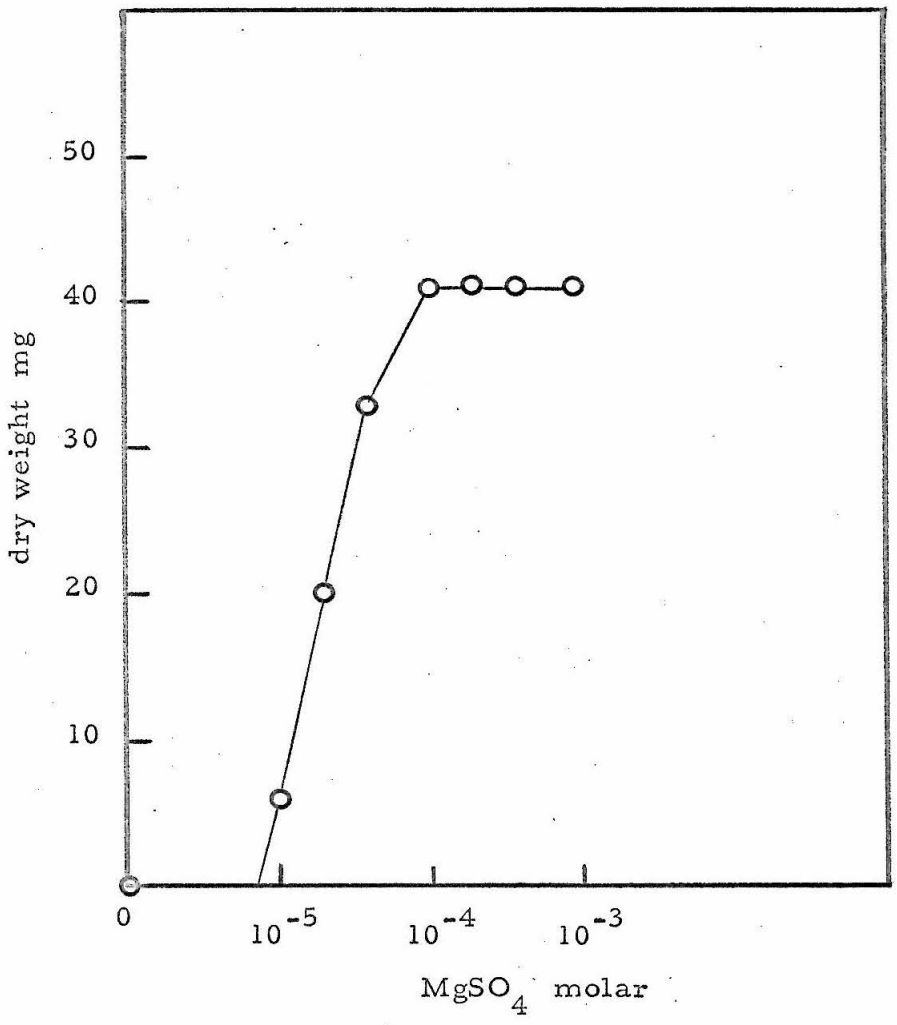


Figure 5. Growth curve of Neurospora for magnesium. 69-1113a was grown for two days on Vogel's minimal medium N with concentrations of $MgSO_4$ from none to 10^{-3}M. Cultures were harvested, dried overnight at 100°C, and weighed. $MgSO_4$ concentration of normal Vogel's minimal medium N is 8×10^{-4}M.

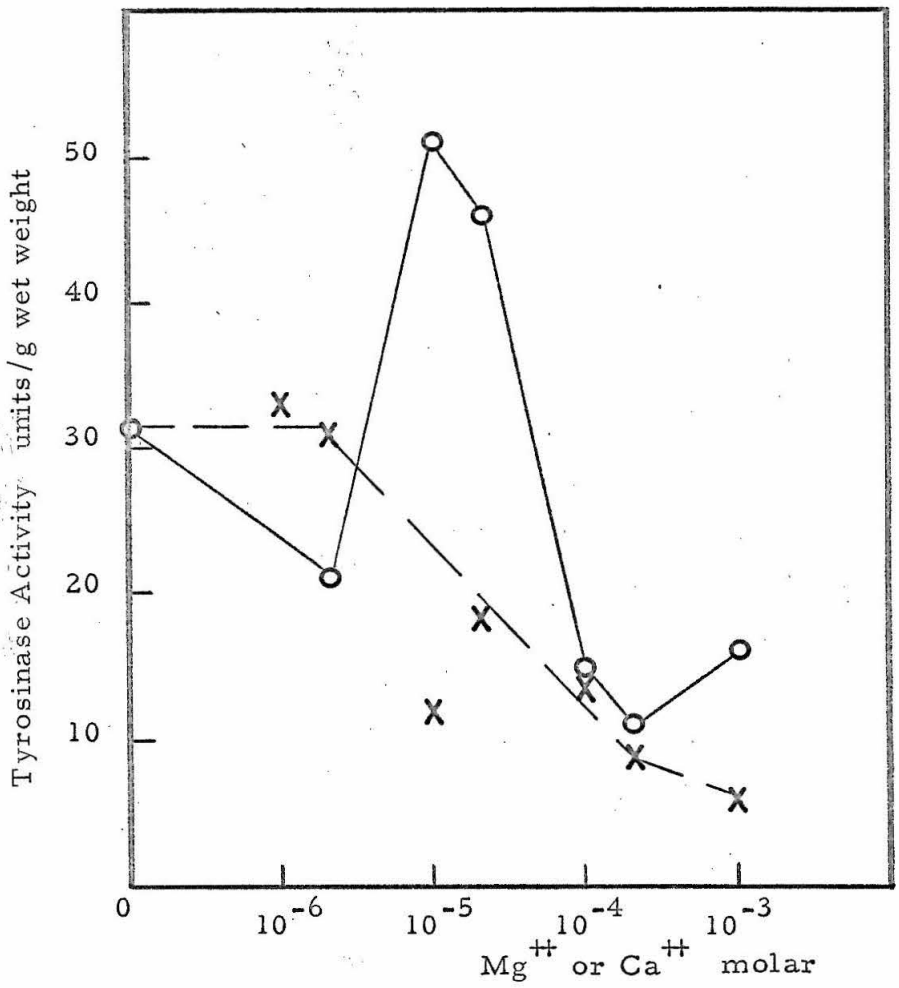


Figure 6. Magnesium vs calcium in the restoration of tyrosinase induction after EDTA. Two-day-old cultures of 69-1113a grown on low Mg^{++} Vogel's medium N (6×10^{-5}M $MgSO_4$) were treated with EDTA and induced in .02M $NaPO_4$ buffer pH 6.0 containing 5×10^{-4}M $CuSO_4$. Either $CaCl_2$ or $MgSO_4$ was added in the molar concentrations shown.

X---X $MgSO_4$ O—O $CaCl_2$

figure 5 shows that magnesium becomes limiting to growth between 6 and 10 x 10^{-5} gm ions/liter. Thus the concentration of magnesium in Vogel's basal salts (8 x 10^{-4} gm ions/liter) is about 10-fold greater than the minimal amount needed to support optimal growth.

Cultures of 69-1113a were grown for two days on low magnesium (8 x 10^{-5}M) Vogel's minimal medium and then treated with EDTA. Copper and L-amino acids were added to all the cultures. In addition, magnesium or calcium was added in concentrations from 10^{-6}M to 10^{-3}M. The results (figure 6) show that magnesium did not stimulate the restoration of tyrosinase synthesis at any concentration, whereas calcium did at about 10^{-5}M. Thus even when cultures were grown on media containing the minimal amount of magnesium to give normal growth, the restoration of tyrosinase induction did not require magnesium. Thus EDTA treatment did not make the induction of tyrosinase dependent on added magnesium.

I. Calcium, Copper, Nickel, and the Growth of Neurospora.

The amount of calcium chloride used to restore induction (5 x 10^{-5}M) is one-tenth that present in Vogel's minimal medium N. On the other hand, 5 x 10^{-4}M cupric sulfate is 500 times the amount present in Vogel's medium N, and no nickel chloride is present in that medium. What changes, if any, do these different cation

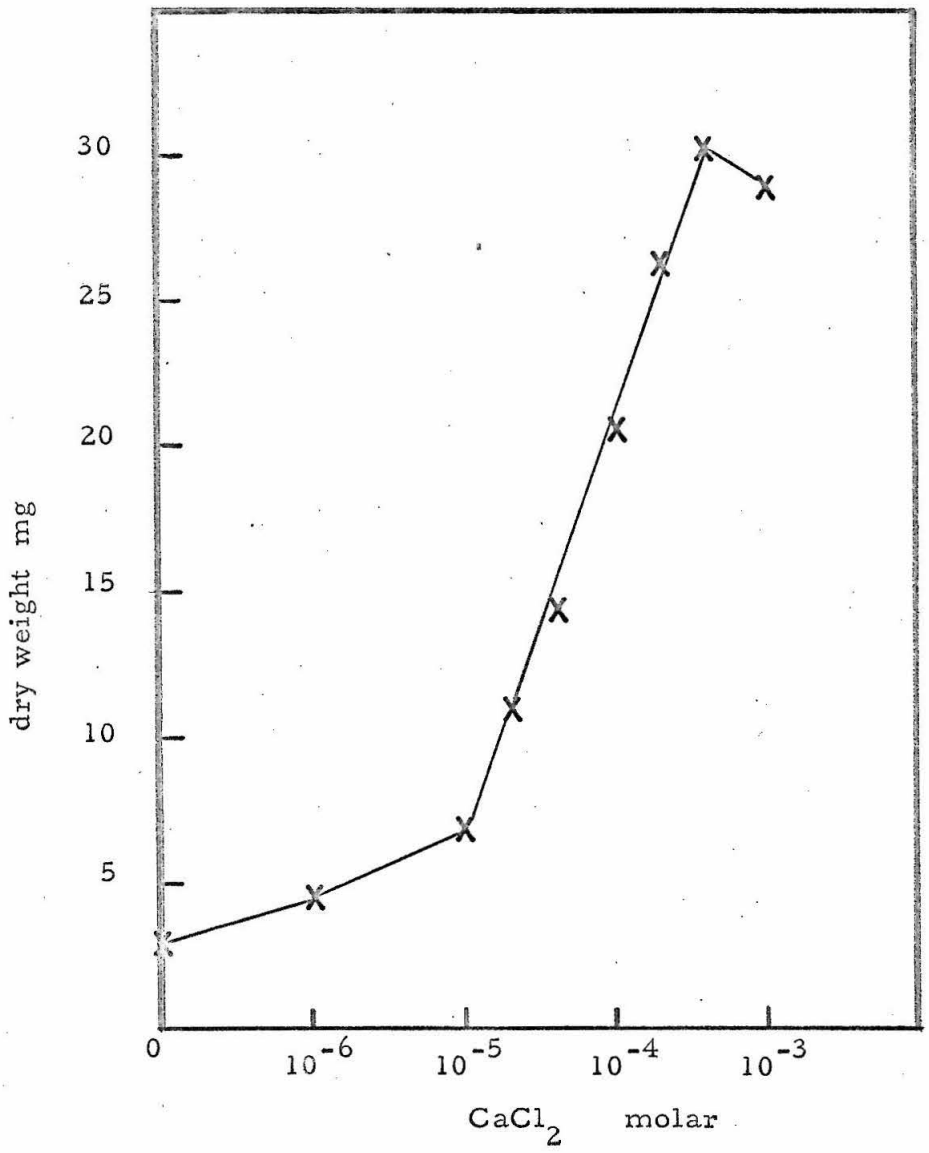


Figure 7. Calcium growth curve of Neurospora. 69-1113a was grown for two days on Vogel's minimal medium N with concentrations of calcium chloride from none to 10^{-3}M. Cultures were harvested, dried overnight at 100°C, and weighed. Normal concentration of calcium chloride in Vogel's minimal medium N is 6.8×10^{-4}M.

concentrations have on the growth and enzyme content of 69-1113a?

A growth curve of Neurospora for calcium chloride is shown in figure 7 . Cultures of 69-1113a were grown on 20 ml of medium N containing zero to 10^{-3}M calcium chloride for two days at 25°C in standing culture in 125 ml Erlenmeyer flasks. The cultures were harvested, dried overnight, and weighed. The figure shows that Vogel's medium N contains the amount of calcium chloride which sustains optimal growth, 6×10^{-4}M. The amount of calcium chloride used to restore induction in the presence of copper, 5×10^{-5}M, is also about that giving half maximal growth. The growth rate for 69-1113a is much more sensitive to the amount of calcium chloride in the medium than the final weight. Thus if the growth experiment was allowed to run for four days before the cultures were harvested, the dry weight of all cultures grown on more than 6×10^{-6}M calcium chloride was the same.

An attempt was made to determine whether copper is essential to the growth of Neurospora, and whether excess copper inhibits growth. Vogel's medium N containing sucrose, but without citrate, was treated with dithezone according to the method of Olson and Johnson (72) to remove any copper in it, and a trace element solution lacking copper was added. Copper chloride was added in concentrations ranging from none to 10^{-2}M. The medium was placed in flasks and

autoclaved. The flasks were inoculated with conidia of 69-1113a and grown without shaking for four days at 25°C. Then the mycelia were harvested, dried overnight at 100°C, and weighed. No mycelium appeared in the medium containing 10^{-2}M copper chloride. Table 28 shows the results of duplicate flasks. The dithezone treatment did

Table 28 . The Effect of Cupric Sulfate Concentration on the Dry Weight of 69-1113a.

Molarity $CuSO_4$	Dry Weight u/g
None	19.9
	27.7
	27.1
	26.6
10^{-6}	30.0
	23.9
10^{-5}	33.3
	31.1
10^{-4}	26.2
	26.7
10^{-3}	12.5
	12.1
10^{-2}	---

69-1113a was grown without shaking for two days at 25°C in modified Vogel's medium N (no sodium citrate, no copper sulfate). Copper sulfate added in amounts noted. The cultures were harvested, dried overnight at 100°C, and weighed.

not reveal a requirement for copper. The presence of 10^{-3}M copper chloride inhibited growth, and 10^{-4}M copper chloride did not, although the pads appeared browned. Nicholas (88) was able to

demonstrate a copper requirement in Neurospora on the basis of the dry weight of the mycelium. He also found that 4×10^{-5}M copper chloride was toxic. This difference in toxicity may be due to a strain difference (he used Em 5297a) or to the difference in the media used; his had a pH of 4.8 instead of the 6.0 found in Vogel's medium N. For whatever reason, the amount of copper used in restoring induction (5×10^{-4}M) only slightly inhibited the growth of 69-1113a. Cultures grown four days on different concentrations of copper sulfate all contained the same amounts of tyrosinase, L-amino acid oxidase, and NADase, so the amount of copper used to restore induction did not induce growing cultures of 69-1113a.

Nickel chloride did not inhibit the growth of 69-1113a in Vogel's medium N containing citrate at concentrations up to 10^{-3}M.

J. Heat Activated "Protyrosinase" in EDTA-Treated Cultures.

Tyrosinase can exist in a form which is inactive until heated. This "protyrosinase" was shown by Gest and Horowitz (51) to be completely activated by heating extracts in sodium phosphate buffer for $1\frac{1}{2}$ minutes at 59°C. The extracts of cultures treated with EDTA and then induced in buffer plus the addition of cations and amino acids, were heat activated to reveal any "protyrosinase" in them. As seen in table 29 , only the cultures containing 3×10^{-4}M calcium chloride and 2×10^{-3}M amino acids (each) contained any

"protyrosinase." This amount does not invalidate the conclusions on the optimal amounts of ions necessary to get maximal production of active enzyme after EDTA.

Table 29. Heat Activation of Tyrosinase in Extracts of EDTA-Treated Cultures.

Additions to induction buffer	Ty'ase unheated u/g	Ty'ase heated u/g	Percent increase
Water-washed control	64	60	- 6
EDTA-treated			
None	2	0	(-100)
Low calcium, copper	39	32	-18
Low calcium, copper, L-amino acids	80	74	- 8
High calcium, copper	18	14	-22
High calcium, copper, L-amino acids	72	96	+33

Two-day-old cultures of 69-1113a treated with EDTA and induced for two days in .02M $NaPO_4$ buffer pH 6.0 with the additions noted.
Low $CaCl_2$ 3×10^{-5}M; high $CaCl_2$ 3×10^{-4}M
$CuSO_4$ 3×10^{-4}M; L-amino acids 2×10^{-3}M each

K. The Effect of EDTA Treatment on the Metal Ion Content of Neurospora.

One expected result of treating the mycelium with EDTA was a removal of large amounts of cations from it. To test this

prediction, duplicate cultures were prepared, one set was washed with EDTA and the other set with 0.1M sodium phosphate without EDTA. Both samples were washed twice with deionized water, and then combusted at 450°C in a muffle furnace in porcelain crucibles. Table 30 shows the metal content of the ash determined in the Spectrographic Laboratory of the Division of Geology, and the tyrosinase content of duplicate cultures to those ashed.

Table 30. Metal Content of Ash of Cultures Treated with EDTA or With Buffer.

Washed with	Amount %						Ty'ase[1]
	Ca	Cu	Fe	Mg	Zn	Al	u/g
.10M $NaPO_4$ pH 6.0	0.2	0.04	0.3	1.[2]	0.4	0.5	162
.5% EDTA in .10M $NaPO_4$ pH 5.6	0.3	0.04	0.3	1.[4]	0.4	0.2	1.0

Two-day-old cultures of 69-1113a were washed with either 0.5% EDTA in .10M $NaPO_4$ buffer pH 5.6 or .10M $NaPO_4$ buffer pH 6.0 without EDTA. Each group was rinsed 2X with deionized distilled water and then ashed in porcelain crucibles at 450°C in an electric furnace. Enough mycelium was used to obtain at least 100 mg ash. Analyses by flash spectrography.

[1] Tyrosinase content of cultures which were duplicates of those ashed.

The most striking feature of the results is that the amount of calcium, copper, and magnesium was virtually the same in the two

ashes. Thus it appears that EDTA has removed so little of these three metals that the difference cannot be detected. This leaves two alternative explanations of how EDTA inhibits induction: (1) some EDTA is left in the mycelium and complexes certain essential cations; (2) the EDTA removes a small but essential fraction of the cations present. The results with nickel and copper suggest that EDTA acts by method #1. The fact that in the presence of copper or nickel full induction is restored only if calcium is added suggests that EDTA has removed a small fraction of the calcium of the mycelium.

L. The Effect of Water Washings on Induction.

If EDTA is merely removing loosely bound calcium from the outside of cells, then it might be possible to duplicate its effect by repeated water washings. This was tried once as shown in table 31.

Table 31 . Effect of Multiple Water Washes of Induction of the Three Enzymes.

Washed with water	Ty'ase u/g	L-Ox'ase u/g	NADase u/g
2 times	62	1.07	35.4
10 times	98	1.34	16.4

Two-day-old cultures of 69-1113a were washed with water either 2X or 10X and induced for two days in .02M $NaPO_4$ buffer pH 6.0.

As can be seen, washing the mycelium 10 times with water instead of twice increased the yield of tyrosinase, did not affect the yield of L-amino acid oxidase, and halved the yield of NADase during the subsequent induction. Thus a five-fold increase in the number of water washes did not mimic the effect of EDTA on induction.

M. Effect of Sugars on the Restoration of Induction in EDTA-Treated Cultures.

When cultures of Neurospora are starved in buffer, they produce large amounts of tyrosinase, L-amino acid oxidase, and NADase despite the absence of an energy source in the buffer. Presumably, the glycogen present in these cultures is used as the energy source for these syntheses (66). The enzyme α-amylase, which breaks down glycogen, has been purified from many organisms (67). It is a calcium requiring enzyme. Although it is usually very difficult to remove all the calcium from the enzyme, it was possible that EDTA was removing enough to make it unstable, thereby inactivating it. Therefore, it is possible that EDTA could inhibit the utilization of glycogen by starving cultures. To test this explanation, three sugars--sorbose, raffinose, and glucose--were added to EDTA-treated cultures in the presence of copper and amino acids (table 32).

Table 32. Effect of Sugars on the Restoration of Tyrosine Induction After EDTA-Treatment.

Additions to induction buffer	Ty'ase u/g
Water-washed control	62
EDTA-treated:	
Ca^{++}, Cu^{++}, amino acids	120
Cu^{++}, amino acids	66
Cu^{++}, amino acids + sorbose	7.6
Cu^{++}, amino acids + raffinose	18
Cu^{++}, amino acids + glucose	2.2
Ca^{++}, Cu^{++}, amino acids + glucose	22

Two-day-old cultures of 69-1113a were treated with EDTA and placed in .02M $NaPO_4$ buffer pH 6.0 with the additions noted: $CaCl_2$ 5×10^{-5}M; $CuSO_4$ 5×10^{-4}M; L-amino acids 2×10^{-3}M each; sorbose, raffinose and glucose, 1% each.

At the concentration tried, all three sugars inhibited recovery rather than stimulating it, as would be expected if they were capable of replacing calcium. This result does not allow any decision to be made about the hypothesis of EDTA action made in this section.

N. The Effects of EDTA Treatment on the Mycelium.

In order to understand better how EDTA inhibits induction, several studies were undertaken on the effects of EDTA on the properties of the mycelium.

0. The Effect of EDTA on Dry Weight During Induction.

During the induction of tyrosinase in phosphate buffer, the dry weight of the mycelium decreases. The leakiness of EDTA-treated cultures suggested that EDTA treatment would increase this weight loss. The decrease in dry weight and its relation to additions to the buffer after EDTA treatment was therefore determined. It was hoped that some relationship between effect on weight loss and the restoration of induction would be detected. Two-day-old cultures of 69-1113a were treated with EDTA and placed in the appropriate induction buffer. During the next 50 hours, cultures were harvested, dried overnight at 80°C, and weighed. Figure 8 shows the change in dry weight with time of these cultures. Duplicate 50-hour cultures were harvested and their tyrosinase content measured and recorded in table 33 .

Several points are demonstrated by these data: First, both water-washed and EDTA-treated cultures had lost a large fraction of their original unwashed mass at the start of induction, 16% and 23%, respectively. Second, theshapes of the control curve, the EDTA-treated phosphate only curve, and the EDTA-treated Vogel's salts curve were very similar. Third, after 50 hours, the dry weights ranged from 68% down to 52% of the original dry weight. Fourth, there was no correlation between the dry weight of a 50-hour culture

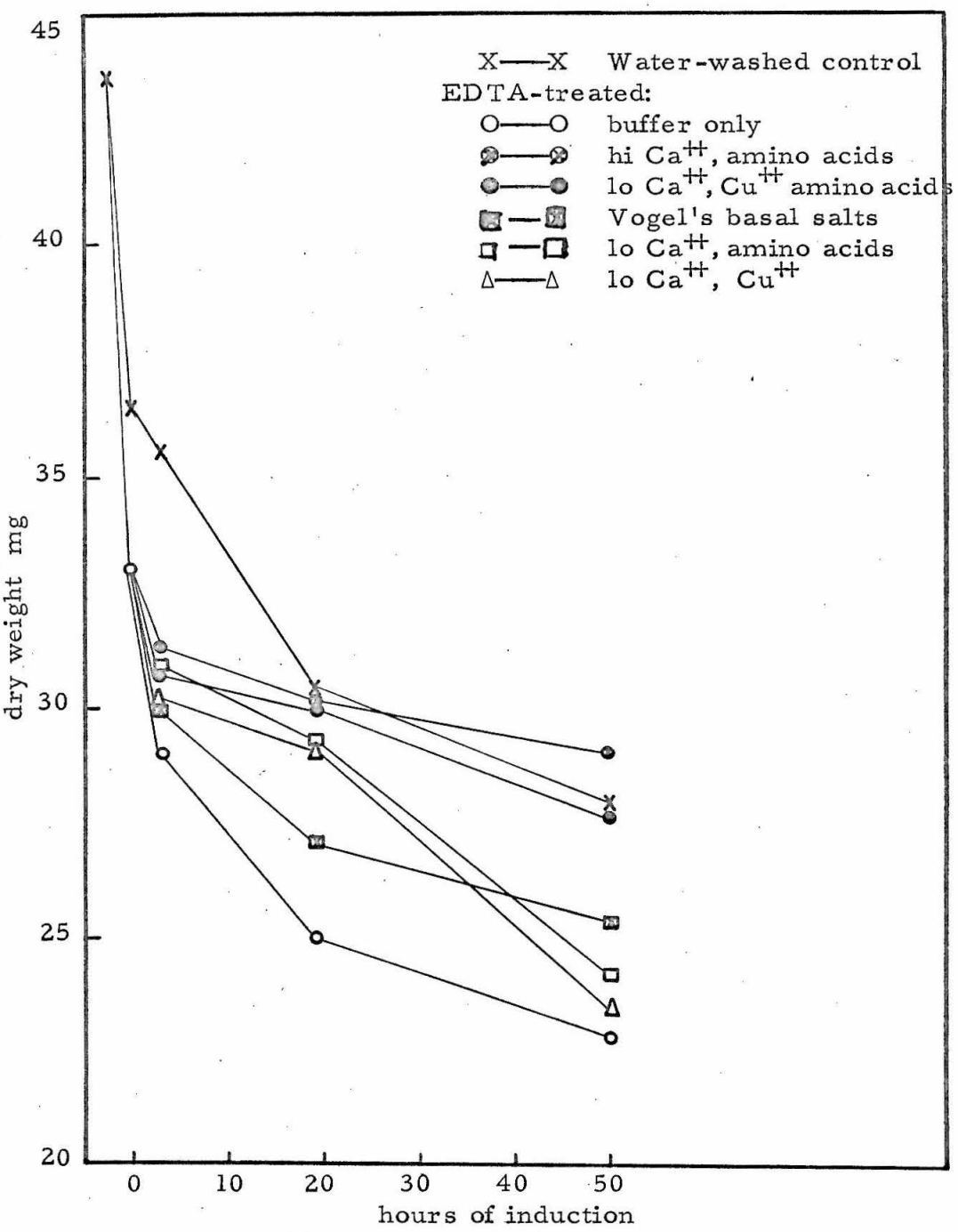


Figure 8. The decrease in dry weight during induction. Two-day-old cultures of 69-1113a were treated with EDTA and placed in .02M $NaPO_4$ buffer pH 6.0 with the additions noted in table 33. They were harvested, dried overnight at 100°C and weighed.

Table 33. The Dry Weight and Tyrosinase Activity of EDTA-Treated Cultures.

Additions to buffer	Dry weight mg	% original dry weight	Ty'ase u/g
Water-washed control	27.9	64	156
EDTA-treated:			
None-EDTA control	22.8	52	1.1
Low calcium, copper	23.5	54	146
Low calcium, copper, L-amino acids	27.6	63	195
Low calcium, L-amino acids	24.2	56	9
High calcium, L-amino acids	29.0	68	232
1X Vogel's basal salts[1]	25.4	58	76

Two-day-old cultures of 69-1113a were treated with EDTA and induced 50 hours in .02M $NaPO_4$ buffer pH 6.0 with the additions noted: The dry weights were determined on duplicates of the cultures used to determine tyrosinase activity.
$CuSO_4$ 5×10^{-4}M; high calcium 5×10^{-4}M $CaCl_2$; low calcium 5×10^{-5}M $CaCl_2$; L-amino acids 2×10^{-3}M each.

[1] Vogel's salts instead of buffer.

and the amount of tyrosinase in its duplicate. Fifth, addition of Vogel's medium N to EDTA-treated cultures starved for 50 hours in buffer caused a resumption of growth, showing that at least part of the mycelium was still alive at that time.

P. An Increase in Mycelial Permeability After EDTA Treatment.

One role of calcium in cells is to stabilize membranes, making both depolarization and changes in ion permeability less likely to occur (89). Since calcium is required to restore induction, it was thought that EDTA might be affecting the cell membrane by increasing its permeability. Recently Leive (68,86) has shown that a brief EDTA treatment does increase the permeability of E. coli in a non-specific manner.

One consequence of increased cell permeability might be the loss of amino acids and nucleosides to the medium resulting in inhibition of induction due to a lack of precursors of the enzymes. Therefore, the various wash solutions from control and EDTA-treated cultures were studied to determine the amount of material absorbing at 260 mμ in them. Figure 9 shows the absorption spectrum of fresh Vogel's medium N and medium in which 69-1113a had grown for two days. The old medium, curve B, has a definite shoulder at 260 mμ , suggesting the presence of nucleosides in the medium. Figure 10 shows the absorption spectra of the water used to wash the control cultures and of a 1: 5 dilution of two-day-old medium (curve C). Two points are important: (1) the first water wash, curve A, contains more absorbing material than curve B, although the volumes of the two washes and their time in contact

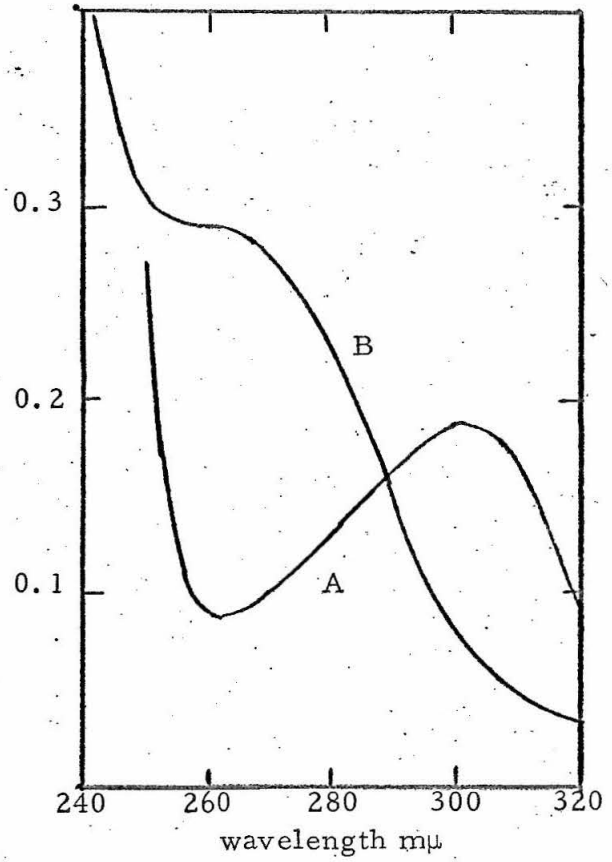


Figure 9. Absorption spectra.
A. Fresh Vogel's medium N vs water blank.
B. Vogel's medium N after 69-1113a has grown in it for 2 days vs fresh Vogel's medium N.

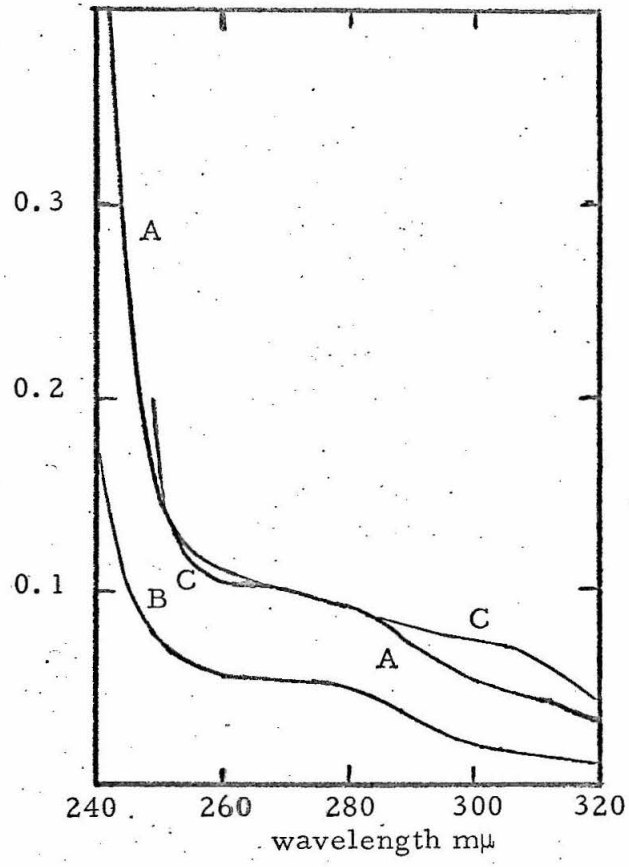


Figure 10. Absorption spectra.
A. 1st water wash of control mycelium vs water blank: vol. 10 ml; time in contact with mycelium 30 min.
B. 2nd water wash of control mycelium vs water blank: vol. 10 ml; contact time 30 min.
C. 2-day-old Vogel's medium N diluted 1:5 vs. water blank.

with the mycelium were the same; (2) both curves resemble closely the diluted old medium. Figure 11 shows the absorption spectra of EDTA (curve A), the two EDTA washes of the mycelium (curves B and C), and the two water washes used to remove the excess EDTA from the treated mycelium (curves D and E). First notice that each wash contains material absorbing at about 260 mμ. In contrast to the control cultures, each wash contains more of this material than the preceding wash. The amounts of material in these washes can be roughly quantitated by assuming that they are an equimolar solution of the four common ribonucleosides; such a solution should have a millimolar absorbancy of about 10. Table 34 shows the amount of ribonucleosides present in each wash solution, as estimated using these assumptions. Thus EDTA does increase the permeability of the mycelium.

It was decided to follow the permeability of cultures during induction to determine whether control cultures become as permeable as EDTA-treated cultures. Two-day-old cultures of 69-1113a were treated with EDTA or water-washed and induced. Figures 12 and 13 show the absorption spectra of the induction buffer after 2 hours 20 minutes and 17-1/2 hours of induction. The water-washed controls do lose 260 mμ absorbing materials to the buffer, but much later in the course of induction. The addition of calcium and copper,

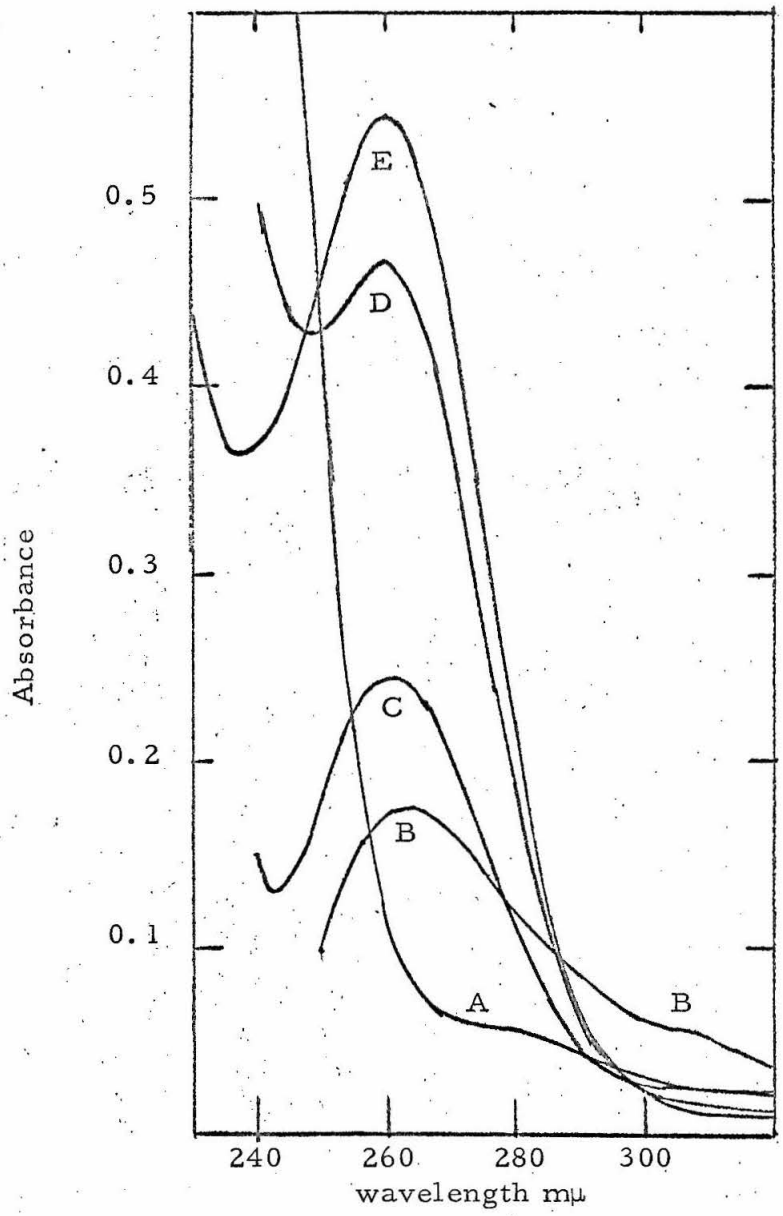


Figure 11. Absorption spectra.

A. 0.5% EDTA in 0.1M $NaPO_4$ buffer pH 6 vs water blank.

B. First EDTA wash of mycelium vs EDTA blank:
vol. 10 ml; time in contact with mycelium 20 min.

C. Second EDTA wash of mycelium vs EDTA blank:
vol. 10 ml; contact time 20 min.

D. First water wash of EDTA-treated mycelium vs water blank:
vol. 10 ml; contact time 30 min.

E. Second water wash of EDTA-treated mycelium vs water blank:
vol. 10 ml ; contact time 30 min.

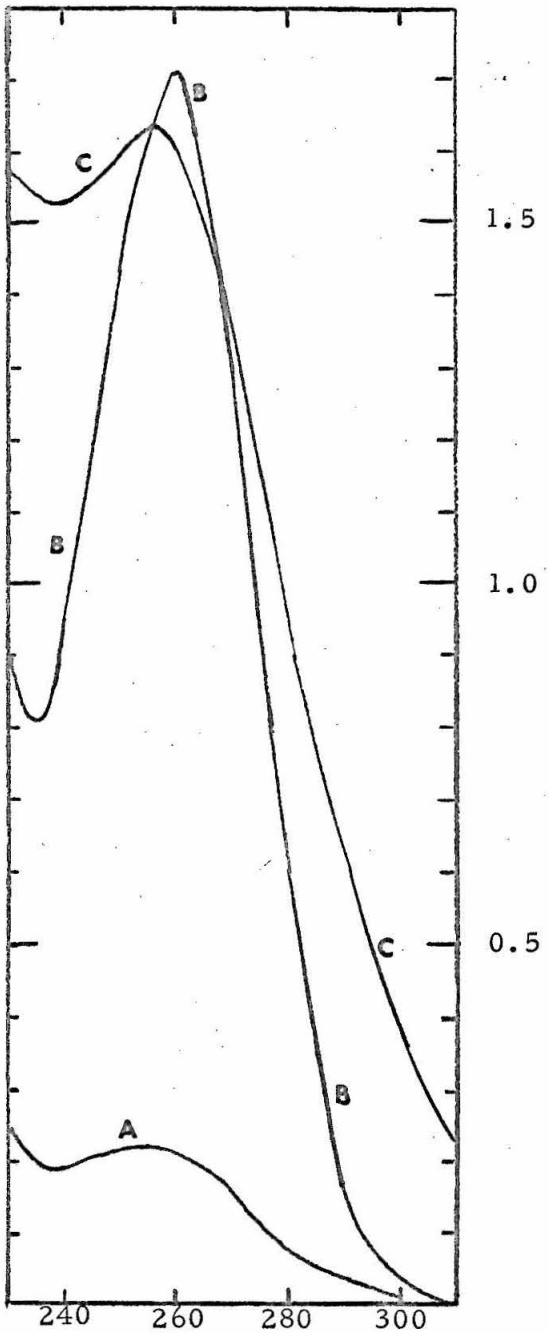


Figure 12. Absorption spectra of $2\frac{1}{2}$ hour induction buffers.

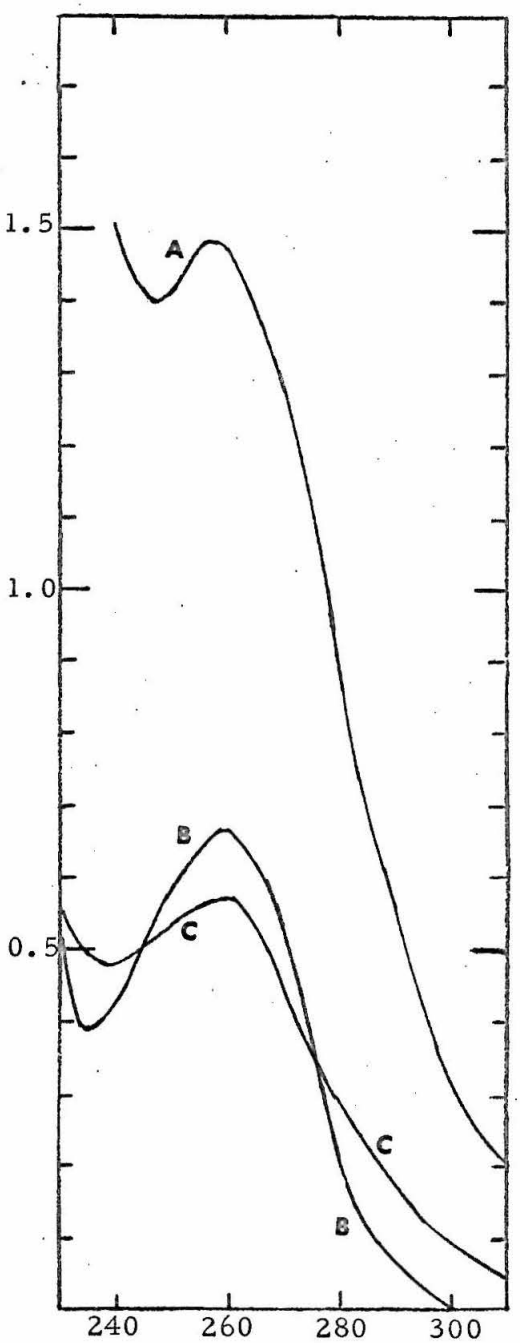


Figure 13. Absorption spectra of $17\frac{1}{2}$ hour induction buffers. Samples B & C diluted 1:5.

Curve A: water-washed control. Curve B: EDTA-treated control. Curve C: EDTA-treated buffer + 5×10^{-5}M $CaCl_2$ + 5×10^{-4} $CuSO_4$.

which should partially restore induction, did not decrease the permeability of the EDTA-treated mycelium. Table 35 shows the estimated amounts of ribonucleosides lost to the induction buffer by these cultures.

Table 34 . Amount of Ribonucleosides in Each Wash Solution of an EDTA-Treated Culture.

Solution	Concentration of ribonucleosides [1] mμM/ml
1st EDTA wash	18
2nd EDTA wash	24
1st water wash of EDTA-treated	46
2nd water wash of EDTA-treated	60

For details on preparation, see figure 11 . Ribonucleoside content estimated from A_{260} and the assumption that the millimolar absorbancy of an equimolar solution of ribonucleosides at pH 6 is 10. [1]Ribonucleoside nature of material arbitrarily assumed.

Table 35. The Amount of Ribonucleosides in the Induction Buffer.

Time	Treatment	Additions to buffer	Concentration of ribonucleoside [1] mμM/ml
2 hr 20 min	Water-washed	None	20
	EDTA	None	170
	EDTA	Ca^{++}, Cu^{++}	160
17-1/2 hr	Water	None	150
	EDTA	None	330
	EDTA	Ca^{++}, Cu^{++}	280

(footnote to table 35)

Two-day-old cultures of 69-1113a were washed with water or EDTA and induced in .02M $NaPO_4$ buffer pH 6.0 with the additions noted. The cultures were harvested at 2 hours 20 minutes or 17-1/2 hours, and the spectrum measured against a water reference. (see figs. 12 & 13) $CaCl_2$ 5×10^{-5}M; $CuSO_4$ 5×10^{-4}M.

[1] Estimated as in table 34.

The water-washed culture loses about 130 mμ M/ml of bases between 2-1/2 hours and 17-1/2 hours, which is about the same as the EDTA-treated cultures. Thus it would appear that in regard to permeability, the difference between the EDTA-treated cultures and the control cultures is mainly that EDTA-treated cultures are leakier earlier, so that at any time the EDTA-treated cultures have lost more material than the water-wash control.

In a separate experiment, shown in table 36 , the amount of ninhydrin positive material accumulating in the induction buffer in 48 hours was measured, as well as the amount of nucleosides. Calcium and copper at levels which restored 50% of induction did not cause a decrease in the amount of amino acids in the buffer, and only slightly decreased the amount of bases.

In summary, these experiments indicate, first, that the water-washed control cultures lose large amounts of material to the buffer, but later than EDTA-treated cultures, and, second, that

Table 36. The Contents of the Induction Buffer of Induced Cultures.

Washed with	Added to buffer	Ty'ase u/g	Ribonucleoside mμ M/ml	Ninhydrin positive as μM ile/ml
Water	None	74	230	3.4
EDTA	None	1	482	4.4
EDTA	Ca^{++}, Cu^{++}	35	372	4.4

Two-day-old cultures of 69-1113a were treated with EDTA or water and induced for two days in .02M $NaPO_4$ buffer pH 6.0 with the additions noted.

$CaCl_2$ 5×10^{-5}M; $CuSO_4$ 5×10^{-4}M

The buffers were saved and the absorption spectrum determined using water as reference. Ninhydrin positive material was determined by the method of Moore and Stein (61), with isoleucine as standard. The concentration is in μ Moles isoleucine/ml.

partial restoration of induction by calcium and copper did not greatly change this increased permeability. The selective permeability of cell membranes depends on active cellular metabolism. The loss of the permeability barrier in starving water-washed cultures is most probably due to the lowered metabolic activity of these cells. It may be that EDTA indirectly increases the permeability of Neurospora by inhibiting metabolism, although Leive (86) has shown this is not the mechanism in E. coli.

Q. The Effect of EDTA Treatment on the Incorporation of Amino Acids Into Proteins in Inducing Cultures

The treatment of cultures with EDTA inhibits the synthesis of three proteins, tyrosinase, L-amino acid oxidase, and NADase during subsequent induction in buffer. The synthesis of two of these enzymes is fully restored by the addition of calcium, copper, and L-amino acids to the induction buffer. Experiments were carried out to determine whether EDTA inhibited uptake of exogenous amino acids and general protein synthesis and, if it did, whether the above additions reversed this inhibition.

Two-day-old cultures of 69-1113a were treated with EDTA and placed in buffer with the additions noted in table 37. Then DL-valine-1-C^{14} was added, and the cultures incubated for 15 minutes. The incorporation was stopped and the amino acids extracted by the procedure of Roberts et al. (40), as described in the Methods section. It is apparent that the EDTA treatment strongly inhibited the uptake of valine from the buffer. In experiment #2, some counts were taken up by the culture treated with EDTA and placed in plain buffer, but these counts in the free amino acid pool did not move into any other fraction, suggesting that EDTA inhibited protein synthesis as well as the uptake of exogenous amino acids. Calcium chloride, at the higher concentration used, restored uptake

Table 37. The Incorporation of Radioactive Valine by EDTA-Treated Cultures.

Additions to induction buffer	Counts recovered in				
	Induction buffer cpm	Cold TCA soluble cpm	Ethanol ether soluble cpm	Hot TCA soluble cpm	NaOH soluble (protein) cpm
Experiment #1:					
Water-washed control	9,500	54,000	1,770	7,000	17,000
EDTA-treated:					
None	113,000	238	84	0	280
Calcium	89,000	2,400	350	880	2,400
Copper	111,000	240	62	0	30
Calcium + copper	112,000	350	56	0	180
Magnesium	104,500	2,500	49	0	30
Experiment #2:					
Control	1,800	28,000	1,570	5,900	12,300
None	56,000	4,250	150	0	104
Low calcium	51,000	15,700	454	446	502
High calcium	----	49,000	2,200	4,200	7,360
Magnesium	38,000	30,000	1,630	1,370	1,800
Copper	71,500	3,000	0	0	135
Low calcium + copper	4,000	4,350	304	0	52

Experiment #1:

Cultures were induced 90 minutes before addition of DL-valine-1-C^{14}. 116,000 counts of DL-valine-1-C^{14} added to each flask. Concentrations: $CaCl_2$ 5×10^{-5}M; $CuSO_4$ 5×10^{-4}M; magnesium 10^{-4}M. Cultures were incubated 15 minutes with shaking (cf. p. 17).

Experiment #2:

Cultures were induced for 30 minutes before addition of DL-valine-1-C^{14}. 84,000 counts of DL-valine-1-C^{14} were added to each flask. Concentrations: low calcium 5×10^{-5}M $CaCl_2$; high calcium 2×10^{-3}M $CaCl_2$; $MgSO_4$ 10^{-3}M; $CuSO_4$ 5×10^{-4}M. Cultures were incubated 15 minutes with shaking.

of the valine from the buffer and partially restored protein synthesis. The lower concentration of calcium was much less effective. Copper alone or in conjunction with the lower concentration of calcium did not restore the uptake of exogenous amino acids. Since the number of counts in the free amino acid pool is very low, it is not possible to conclude definitely that protein synthesis was not restored by this treatment although the data suggest that conclusion. Magnesium did restore uptake and synthesis but was less effective than calcium. The presence of other amino acids completely inhibited uptake of C^{14} valine from the buffer, even though all aliphatic amino acids were omitted. This is not surprising in view of the complex interrelations among different amino acids for transport in Neurospora found by St. Lawrence et al. (65).

R. High Calcium Concentrations and the Restoration of Tyrosinase Induction after EDTA.

The effectiveness of high calcium alone in restoring uptake of exogenous amino acids and protein synthesis led to experiments on its ability to restore the induction to tyrosinase. Two-day-old cultures of 69-1113a were treated with EDTA and induced in phosphate buffer containing the additions noted in table 38 . At the highest concentration of calcium, the amount of tyrosinase made was about one-fifth that in the controls, but the addition of amino acids

Table 38. The Restoration of Tyrosinase Induction by Calcium and Amino Acids.

Additions to buffer			Ty'ase u/g
	$CaCl_2$ M	Amino acids	
Control		(No EDTA)	141 151
EDTA treated	2×10^{-3}	No	29 29
		Yes	32 33
	3×10^{-4}	No	7.2 8.6
		Yes	78 99
	3×10^{-5}	No	3.5 1.0
		Yes	3.7 5.9
	None	None	< 0.5

Two-day-old cultures of 69-1113a were treated with EDTA and induced in .02M $NaPO_4$ buffer pH 6.0 with the additions noted. L-amino acids 2×10^{-3}M each.
Control washed with water and induced in .02M $NaPO_4$ buffer pH 6.0.

had no effect upon the amount of enzyme made. At 3×10^{-4}M calcium chloride, large amounts of tyrosinase were made only in the presence of amino acids, almost two-thirds of the control values. As was known before, very little tyrosinase was made when the calcium concentration was 3×10^{-5}M. Thus when the calcium

concentration was high enough to allow the uptake of exogenous amino acids from the buffer, those exogenous amino acids did not affect the amount of synthesis restored. This paradox may explain the role of amino acids in the restoration of induction after EDTA treatment. The EDTA-treated cells lose large amounts of nucleosides and probably amino acids to the wash solutions and induction buffer at early times in the course of induction. Thus the effective concentration of amino acids in the mycelium is greatly decreased and the cells cannot take up the lost amino acids from the buffer, so protein synthesis cannot occur at the highest efficiency. When amino acids are present in the outside buffer in high enough concentrations, or when the calcium concentration is high enough to restore amino acid uptake, the amino acid concentration in the mycelium can be maintained at a high concentration and protein synthesis occurs efficiently. However, high levels of calcium inhibit induction so that these two competing effects of calcium result in a low yield of tyrosinase at 2×10^{-3} M calcium chloride.

S. Time Course of Induction of Tyrosinase After EDTA Treatment.

As reported above, EDTA treatment greatly increases the permeability of the mycelium. It might also affect the time course of induction. Cultures of 69-1113a were treated with EDTA and

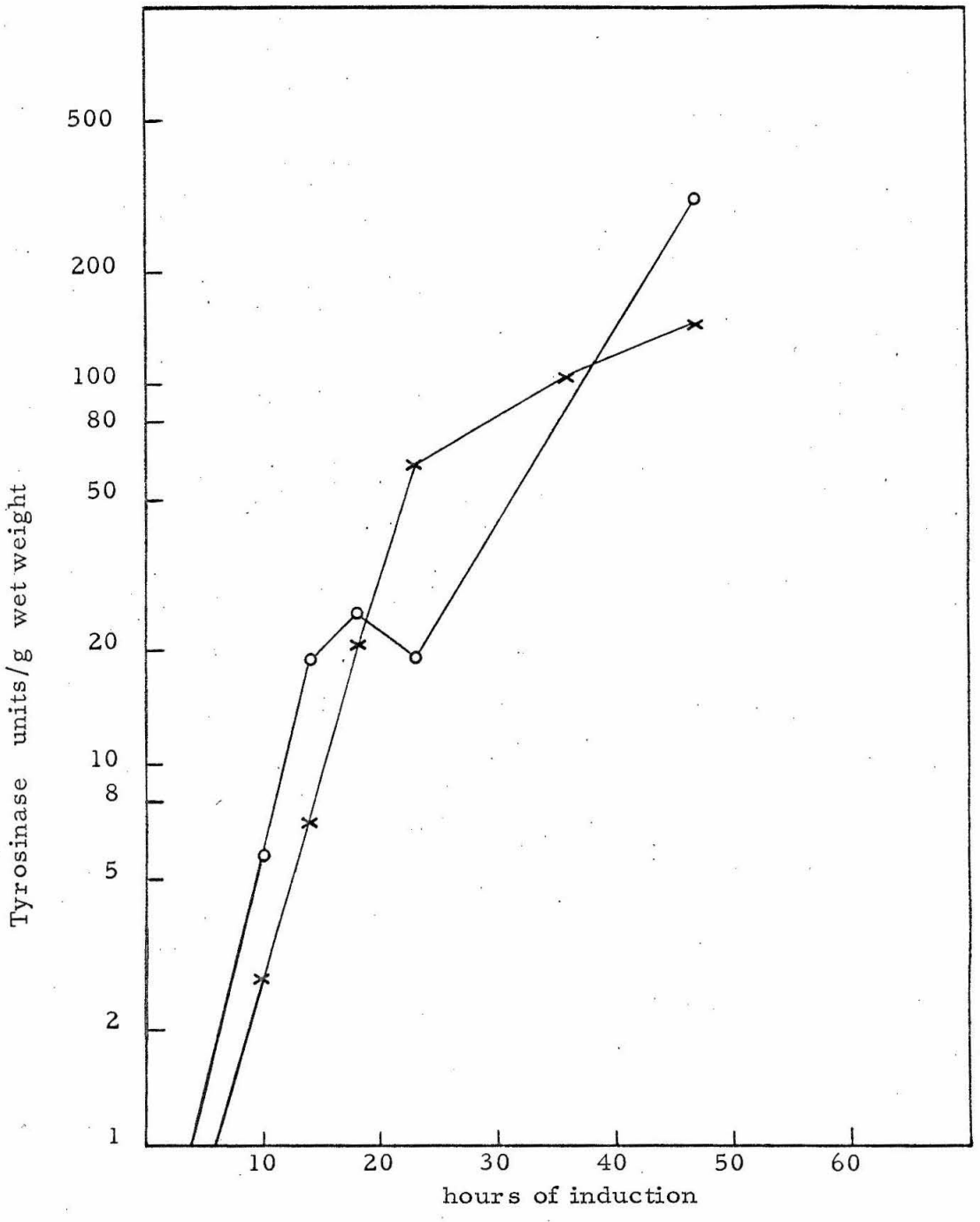


Figure 14. The time course of induction in EDTA-treated and control cultures. Two-day-old cultures of 69-1113a were treated with EDTA and induced in .02M $NaPO_4$ buffer pH 6 containing 5×10^{-5}M $CaCl_2$, 5×10^{-4}M $CuSO_4$, and 2×10^{-3}M L-amino acids. O——O : Control cultures were washed with water and induced in .02M $NaPO_4$ buffer pH 6.0. X——X

induced in phosphate buffer containing calcium, copper, and L-amino acids. Control cultures were washed with water and induced in phosphate buffer without additions. Duplicate cultures were harvested at times over the next 47 hours and their tyrosinase content determined. As can be seen in figure 14, the time course of the EDTA-treated cultures is the same as that of the controls within the error of the experiments. Thus EDTA treatment does not affect the factors determining the time course of tyrosinase induction following restoration by the addition of cations and amino acids.

T. The Requirement for Oxygen During the Lag Period.

The induction of tyrosinase shows three phases: (1) the lag period--the period between the start of induction and the time when enzyme is first detected; (2) the rise phase--the period after the lag phase when enzyme is rising to its maximum concentration due to rapid synthesis; (3) the decay phase--the period when the concentration of the enzyme decreases again.

The lag phase of induction is obviously a dynamic period, since it is the period when conditions change in such a way as to permit tyrosinase synthesis to occur. It has been postulated for several reasons, including the fact that the best inducers are amino acid analogues, that the substance preventing tyrosinase synthesis, the repressor, is a protein which is destroyed during the lag phase.

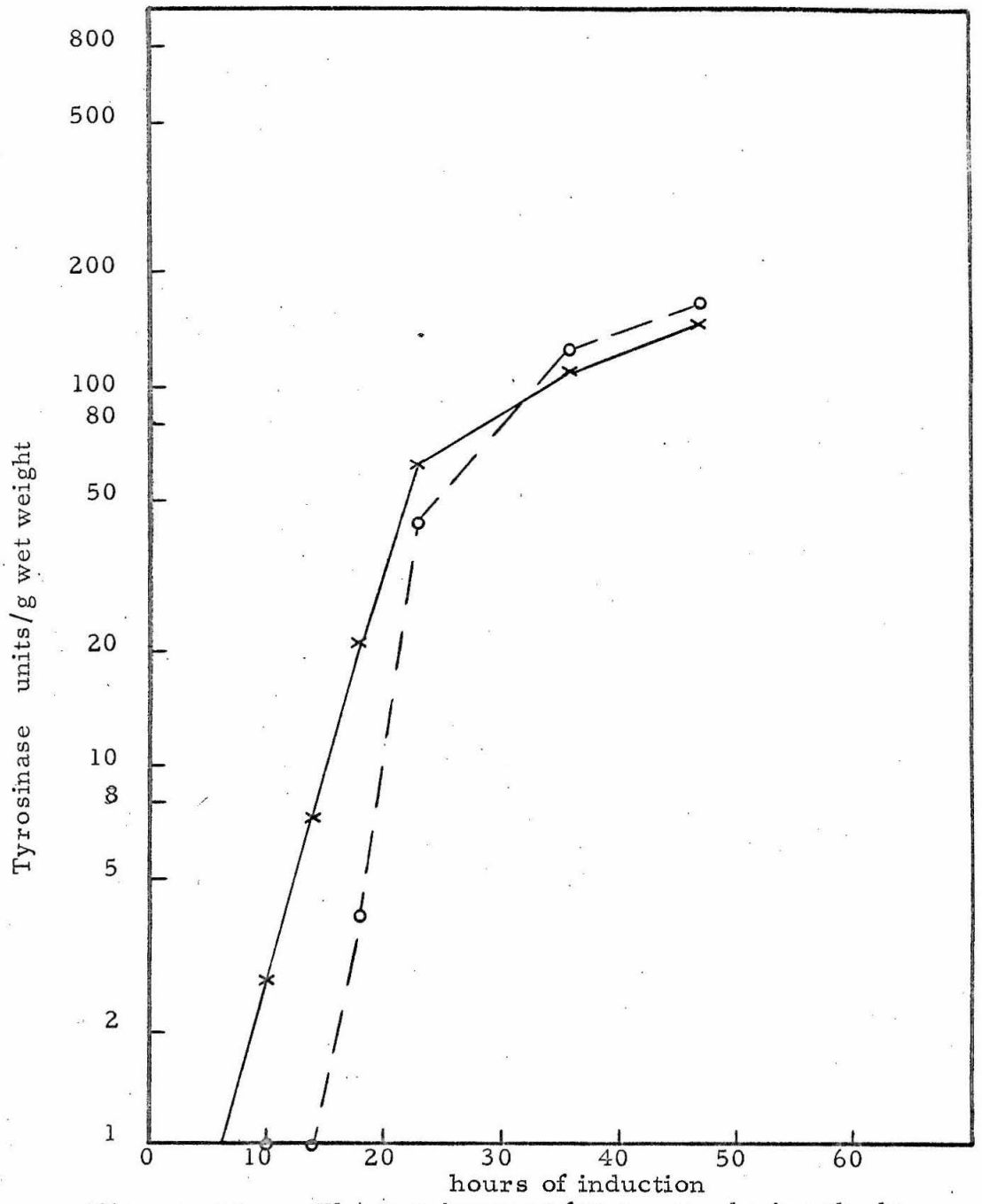


Figure 15. The requirement for oxygen during the lag phase of tyrosinase induction. Two-day-old cultures of 69-1113a were washed with water and induced in .02M $NaPO_4$ buffer pH 6.0. Set #1 air expelled with sterile nitrogen at start of induction; after 10 hours the nitrogen was expelled with air. Set #2 air present during the entire induction. #1 O--O N_2 #2 X——X air

In this case it might be expected that oxygen would not be required during the lag phase, since proteolytic enzymes do not require oxygen in order to function. To test this hypothesis, two sets of cultures were washed with water and put in phosphate buffer. The air in one set was replaced by nitrogen. After ten hours, the nitrogen was expelled with air and duplicate samples were harvested at times thereafter. Figure 15 shows that oxygen is necessary to get through the lag period. Thus the processes involved in the lag period require energy and are more complex than simple proteolysis. In this respect, the events during the lag period resemble protein turnover in mammals, where inhibitors of metabolism and protein synthesis, e.g. N_2 and DNP, inhibit protein catabolism (70, 71). On the other hand, p-fluorophenylalanine is an effective inducer of tyrosinase but inhibits protein breakdown in the mammalian systems (70). Thus the analogy to the mammalian system breaks down. In bacteria, protein turnover in starved cultures appears to be due to simple proteolysis (69), but is eventually inhibited by N_2, DNP, etc. Thus the events during the lag period differ from those involved in protein catabolism in either bacteria or mammals.

U. EDTA and the Lag Period.

As shown in the preceding section, the lag period is a period of dynamic change requiring oxygen. It was of interest to know whether calcium, copper, and amino acids were required

during the lag period. To answer this question two sets of EDTA-treated cultures were prepared. One set had calcium, copper, and amino acids added at the start of induction. The second set was placed in phosphate buffer until nine hours of induction had passed and then calcium, copper, and amino acids were added. If EDTA-treated cultures could pass through the lag phase without cations and amino acids, the second set of cultures should start making tyrosinase immediately when calcium, copper, and acids were added. The results of the experiment are shown in figure 16 . It is evident that the cultures could not get through the lag period after EDTA treatment unless the cations and amino acids were present. In fact, the lag period was increased to 18 hours when the cations and amino acids were not present in the buffer during the first nine hours of induction.

The reason for the increase in the lag period has not been examined experimentally. Three explanations come to mind: (1) the cultures have been damaged by the long starvation in phosphate after EDTA treatment, and this damage must be repaired before the normal processes of the lag phase can proceed; (2) the cultures have gone through the normal processes of the lag phase during starvation in buffer after EDTA treatment, but the absence of ions has imposed new requirements on the system; or (3) the cultures have been

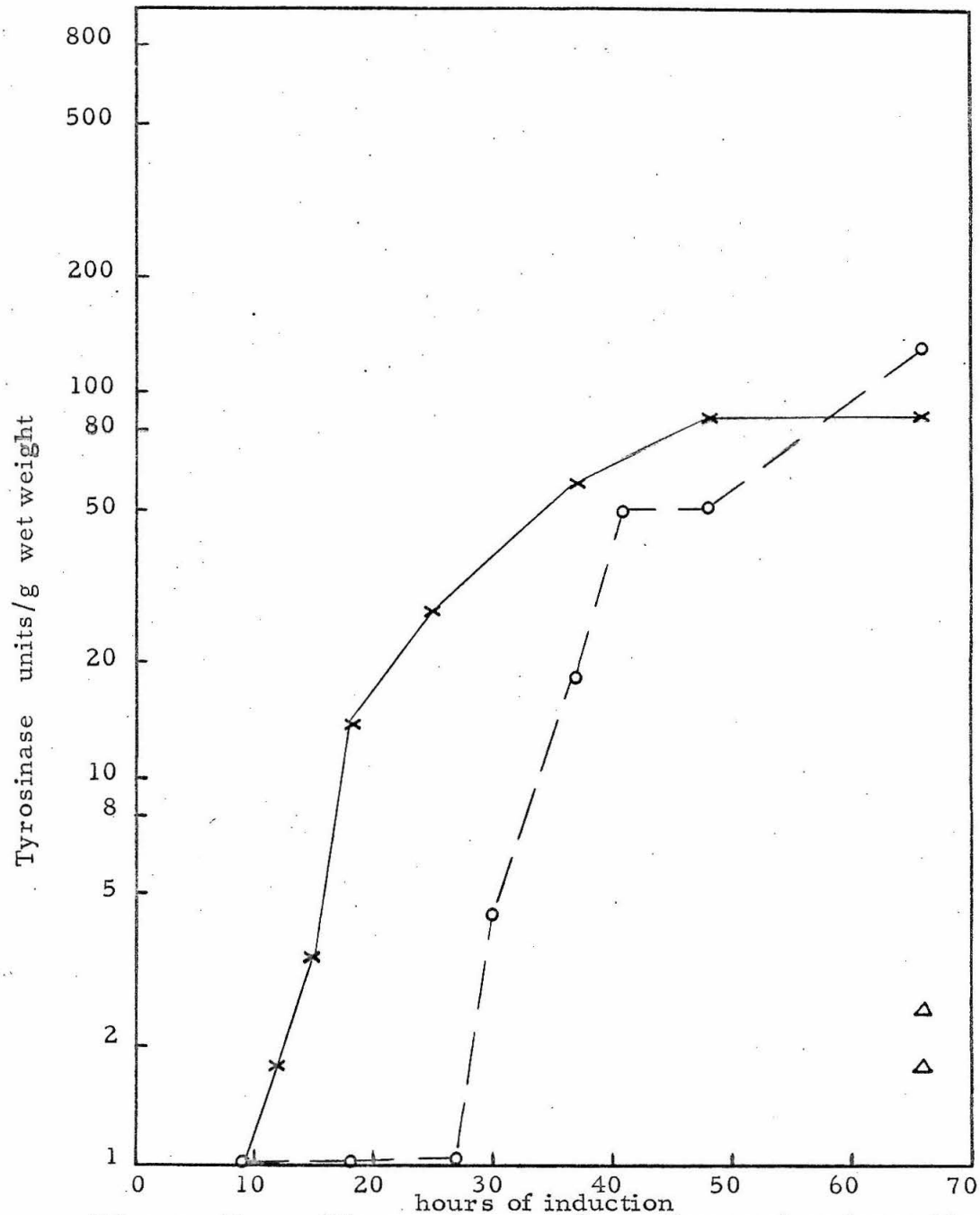


Figure 16. The requirement for cations and amino acids during the lag phase of tyrosinase induction after EDTA treatment. Two-day-old cultures of 69-1113a were treated with EDTA and placed in .02M $NaPO_4$ buffer pH 6.0. Set #1 X——X calcium, copper and amino acids were added at the start of induction. Set #2 O- - -O calcium, copper and amino acids were added 9 hours after the start of induction. Set #3 Δ no additions to the buffer. $CaCl_2$ 5×10^{-5}M; $CuSO_4$ 5×10^{-4}M; L-amino acids 2×10^{-3}M.

depleted of energy during the first nine hours and cannot take up the ions from the buffer as rapidly as at zero time. Dr. Fling in this laboratory has found that after ten hours of starvation in buffer, water-washed cultures do not take up amino acids quickly from the medium (29). Experiments have shown that even at zero time, EDTA-treated cultures take up amino acids very slowly from the medium (cf. p. 89). Thus it seems that a combination of the first and third explanations is the most reasonable one.

V. Completion of the Lag Phase Under Conditions Not Permitting Tyrosinase Production

As shown in the previous section, if no cations nor amino acids are added to the buffer for the first ten hours after EDTA-treatment, the lag phase is prolonged from about 8 hours to about 16 hours. It was decided to determine whether any conditions could be found that would allow the culture to complete the lag phase but not allow it to enter the synthesis phase of induction. Since copper alone gave partial restoration of induction, EDTA-treated cells can obviously complete the lag phase in the presence of copper without added calcium. On the other hand, if the concentration of calcium in the induction buffer was low, no tyrosinase was made after EDTA-treatment. Yet it was possible that in the presence of low calcium the lag phase was completed.

To test this possibility three sets of cultures of 69-1113a were treated with EDTA and placed in buffer. To set #1, calcium, copper, and amino acids were added at time zero to restore induction. To set #2, calcium was added at zero time, and nine hours later copper and amino acids were added. To set #3, nothing was added until the ninth hour, when calcium, copper, and amino acids were added. A set of control cultures were left with calcium only during the entire induction. Duplicate cultures were harvested at different times during induction. Figure 17 shows the time course of each set of cultures. The culture which had calcium present during the lag period was able to complete the lag period as shown by the immediate appearance of tyrosinase upon addition of copper and amino acids. When nothing had been present in the induction buffer during the first nine hours, the lag period was prolonged from less than 9 hours to at least 16 hours after the addition of cations and amino acids, but once synthesis started in these cultures, it was faster than in the other cultures. The culture which had only calcium added to it after EDTA treatment developed less than one unit/g of tyrosinase over 48 hours of induction. Thus EDTA-treated cultures were able to complete the lag phase under conditions which did not allow the production of tyrosinase.

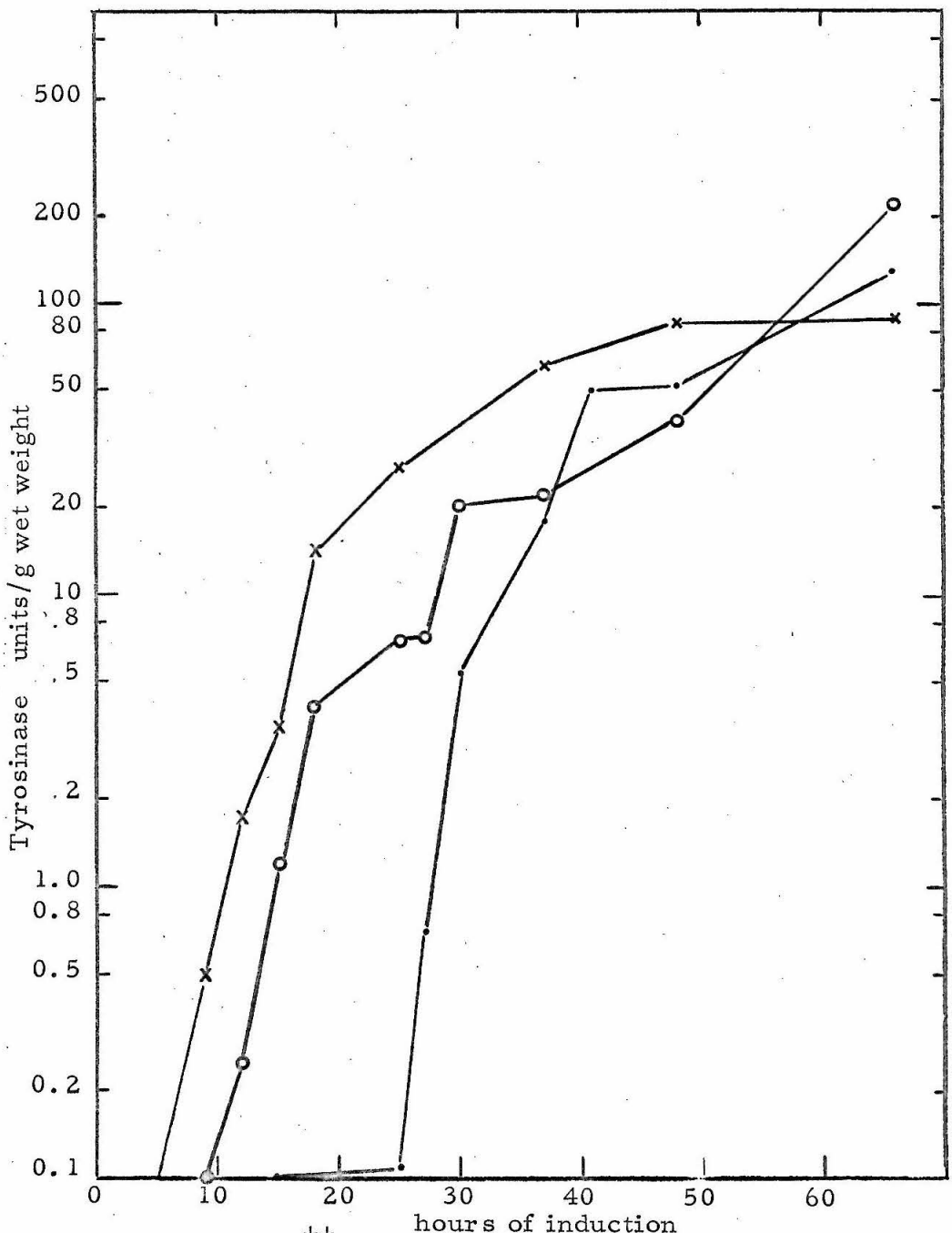


Figure 17. Ca^{++} and the lag phase. Two-day-old cultures of 69-1113a were treated with EDTA and placed in .02M $NaPO_4$ buffer pH 6.0. Set #1: X—X Ca^{++}, Cu^{++}, amino acids added at time zero. Set #2: O—O Ca^{++} added at time zero, Cu^{++}, amino acids added at hour 9. Set #3: ·—· Ca^{++}, Cu^{++}, amino acids added at hour 9. $CaCl_2$ 5×10^{-5}M; $CuSO_4$ 5×10^{-4}M; L-amino acids 2×10^{-3}M (each).

W. The Need for Cations During the Synthesis Phase of Tyrosinase Induction

In the previous section it was shown that after EDTA treatment, the mycelium cannot get through the lag phase unless cations are added to the induction buffer. It is conceivable then that after the cultures are through the lag phase, and in the rise or synthesis phase of induction, the need for cations might not exist. To test this possibility, cultures were allowed to stand in phosphate buffer until they had completed the lag phase and then were treated with EDTA (see table 39). It is clear that EDTA inhibited the synthesis phase as well as the lag phase. The same cations were effective in restoring synthesis as were required in the lag phase. Magnesium at the concentration tested decreased the restoration achieved with copper and amino acids, in sharp contrast to the results with calcium. Thus calcium and magnesium do not affect

Table 39 . Requirements for the Restoration of Tyrosinase Synthesis After the Completion of the Lag Phase.

Additions to buffer	Ty'ase u/g
Water-washed controls	132
EDTA-treated:	
Amino acids	1.7
Ca^{++}, Cu^{++}, amino acids	190
Ca^{++}, amino acids	9
Cu^{++}, amino acids	80
Mg^{++}, amino acids	4.1
Mg^{++}, Cu^{++}, amino acids	66
Culture harvested at time of EDTA treatment	0.75

Two-day-old 69-1113a was washed with water and placed in 5 ml .02M $NaPO_4$ buffer pH 6.0 for 11 hours, then treated with EDTA and returned to new buffer containing the additions noted.
L-amino acids 2×10^{-3}M; $CaCl_2$ 5×10^{-5}M; $CuSO_4$ 5×10^{-4}M; $MgSO_4$ 5×10^{-5}M.
The cultures were harvested 34 hours after the EDTA treatment.

the synthesis phase of induction of tyrosinase in the same way after EDTA treatment.

X. Can ty-1 Perform Any of the Reactions Connected with the Lag Phase in the Absence of Inducer?

The mutant strain ty-1 does not make appreciable amounts of tyrosinase even if it is starved in phosphate buffer for ten days (29).

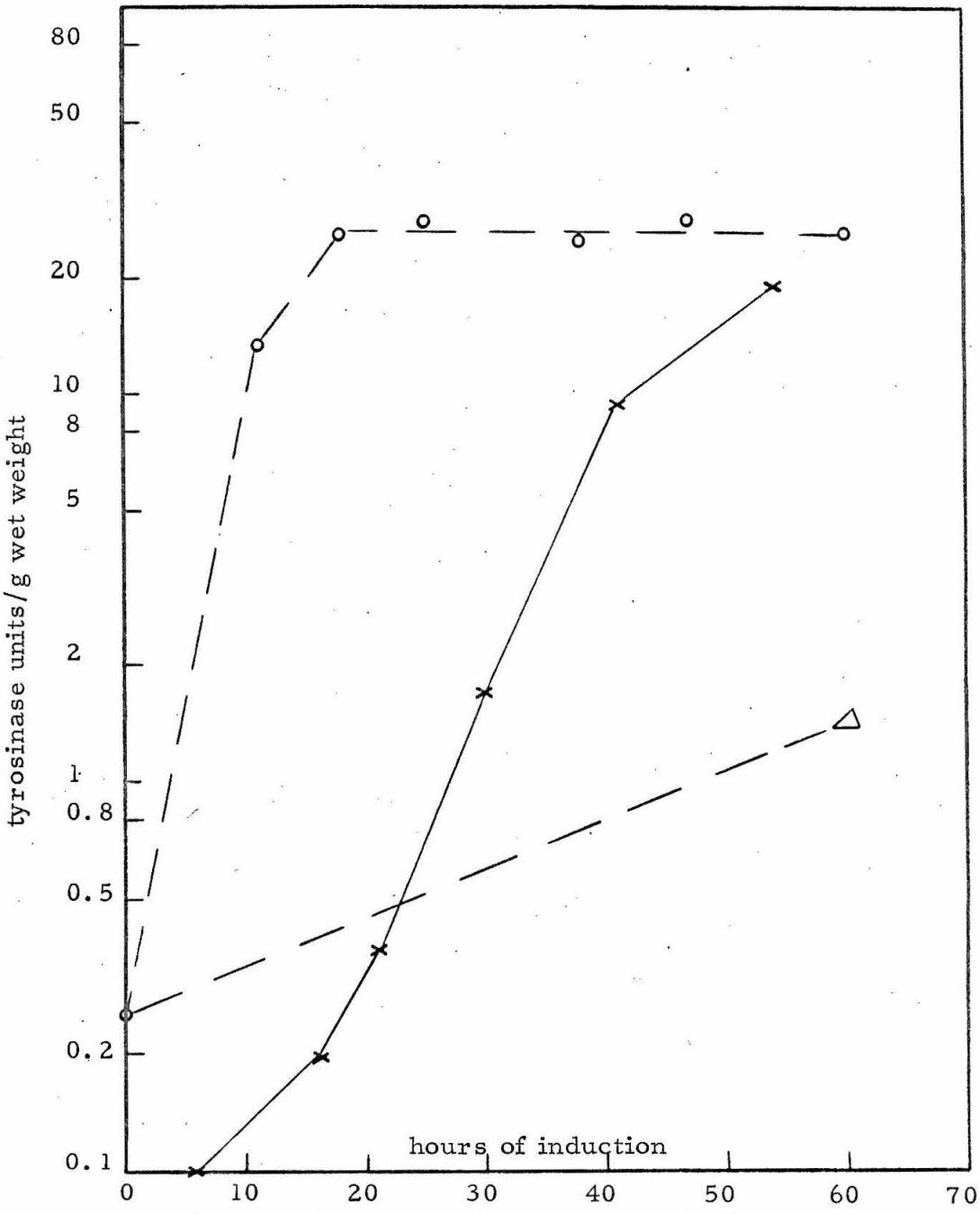


Figure 18. The effect of starvation in buffer without inducer on the time course of a subsequent induction of tyrosinase in the mutant ty-1. Two-day-old cultures of ty-1 were washed with water and placed in .02M $NaPO_4$ pH 6.0. Set #1 had D-phenylalanine 0.2 mg/ml added at day zero. Set #2 had D-phenylalanine added at day $4\frac{1}{2}$. Set #3 did not receive D-phenylalanine. 1. X——X D-Phe at day zero. 2. O---O D-Phe at day $4\frac{1}{2}$. Δ---Δ No-D-Phe.

This result suggests that ty-1 cannot perform some critical step in the series of events occurring during the lag period unless an inducer is present. Another possible explanation was that after ten days, ty-1 could no longer synthesize tyrosinase due to destruction of the synthesizing system. To test these alternatives, two sets of two-day-old cultures of ty-1 were washed with water and placed in phosphate buffer. An inducer, D-phenylalanine, was added to one set immediately, and the time course of induction followed. The second set was allowed to starve for 4-1/2 days before inducer was added. Figure 18 shows the time course of induction in the two experiments. It is obvious that changes had taken place in the starved cultures, which decreased the lag period from about one day to less than eleven hours. These changes did not affect the final amount of tyrosinase made by the cultures. Therefore, ty-1 can perform some of the reactions required to get through the lag period in the absence of an inducer, but not all of them.

Y. Effect of EDTA on Tyrosinase Induction in Ty-1.

When 69-1113a is starved in carbon-free Vogel's minimal medium N for two days, less than 5 units/g of tyrosinase are made in the absence of an inducer. On the other hand, after EDTA treatment, over 50 units/g of tyrosinase are commonly found, without adding an inducer. This result suggests that EDTA treatment can

induce tyrosinase synthesis under these conditions. To test this hypothesis, the mutant ty-1, which requires an inducer for induction in phosphate buffer, was treated with EDTA, and the restoration of induction in the presence and absence of D-phenylalanine measured.

Two-day-old cultures of ty-1 were treated with EDTA and starved in phosphate buffer with the usual additions of calcium, copper, and amino acids. In addition, 1 mg D-phenylalanine was added to half the cultures. Table 40 shows that EDTA did not induce ty-1 to make tyrosinase and that calcium copper and amino acids restored induction of tyrosinase in ty-1 when D-phenylalanine was present. Thus EDTA treatment did not eliminate the requirement for an inducer such as D-phenylalanine to get induction of tyrosinase in ty-1. So EDTA does not affect the same elements of the control systems as inducers affect.

Z. Increased Sensitivity of Induction to Actinomycin D After EDTA Treatment.

Leive has reported that a brief treatment of E. coli cells with 10^{-3}M EDTA renders them completely sensitive to actinomycin (to which they are normally impermeable) without decreasing their viability (68). Neurospora crassa conidia are very susceptible to actinomycin D, but mycelia are much less sensitive (42).

Table 40 . Effect of EDTA on Tyrosinase Induction in ty-1.

Additions to induction buffer	Ty'ase u/g	
	+D-Phe	-D-Phe
Water-washed control	20	0
EDTA-treated:		
None	0	0.5
Cu^{++}	7.6	0.3
Ca^{++}	0.5	0
$Cu^{++} + Ca^{++}$	46	0
Amino acids	0	0.5
Cu^{++} + amino acids	1.6	1
Ca^{++} + amino acids	0	0
$Ca^{++} + Cu^{++}$ amino acids	33	0.5

Two-day-old cultures were treated with EDTA and starved in .02M $NaPO_4$ buffer pH 6.0 with and without 2 mg/ml D-phenylalanine. The other additions to the buffer were 5×10^{-4}M $CuSO_4$, 5×10^{-5}M $CaCl_2$, or 2×10^{-3}M L-amino acids.

After EDTA treatment, the induction of tyrosinase and L-amino acid oxidase is very much more sensitive to inhibition by actinomycin D. (see table 41)

These results confirm the conclusion drawn from earlier work at much higher concentrations of actinomycin D, that both tyrosinase and L-amino acid oxidase induction depend on RNA synthesis during induction.

Table 41. Increased Sensitivity of Induction to Actinomycin D After EDTA Treatment.

Washed with	Additions to buffer	Act D conc. μmolar	Ty'ase u/g	L-Ox'ase u/g	% Inhibition Ty'ase	% Inhibition Ox'ase
Water	None	0	150	3.9		
Water	None	1.25	75	1.5	50	61
EDTA	Ca^{++}, Cu^{++}, amino acids	0	221	4.1		
EDTA	" "	1.25	26	0.45	92	89
EDTA	" "	0.6	30	0.30	90	93
EDTA	" "	0.3	31	0.66	90	84

Two-day-old cultures of 69-1113a were treated with EDTA or washed with water and induced in .02M $NaPO_4$ buffer pH 6.0 with the additions noted. $CaCl_2$ 5×10^{-5}M; $CuSO_4$ 5×10^{-4}M; L-amino acids 2×10^{-3}M each.

AA. The Localization of Tyrosinase and L-Amino Acid Oxidase in the Mycelium.

Metzenberg has determined that the enzyme β-fructofuransidase is located outside the cell membrane of Neurospora (74). It is destroyed by treatment of the mycelium with 0.1N HCl at 0°C for 1 minute, while alkaline phosphatase, an enzyme located inside the cell membrane (75), is only slightly affected. Both tyrosinase and L-amino acid oxidase are completely inactivated by a one minute treatment in extracts with 0.1N HCl at 0°C (table 42). So this test

of Metzenberg may be used to determine whether tyrosinase and L-amino acid oxidase are inside or outside the cell membrane of Neurospora.

Pooled induced mycelia of 69-1113a were treated for 1 minute at 0°C with 0.1N HCl. After neutralizing the acid, the mycelia were ground with sand and extracted as usual. They were assayed for invertase (75), alkaline phosphatase (76), tyrosinase, and L-amino acid oxidase (table 43).

Table 42 . The Inactivation of Tyrosinase and L-Amino Acid Oxidase in Extracts by Acid.

Enzyme	Activity[1] No HCl	+HCl
Tyrosinase	30	0
L-amino acid oxidase	0.401	0.000

[1] Arbitrary units

An extract of an induced culture of 69-1113a was made 0.1N in HCl for 1 minute at 0°C, then neutralized and assayed.

Control mycelia not treated with acid contained all four enzymes. Treated mycelia had lost almost all their invertase activity and L-amino acid oxidase activity. On the other hand, none of the alkaline phosphatase or tyrosinase present was inactivated by the acid treatment. Therefore, L-amino acid oxidase may be located

outside the cell membrane where it can be inactivated by acid. Tyrosinase is probably located inside the cell membrane where the acid cannot reach it.

Table 43. The Sensitivity of Four Enzymes in Whole Mycelia to Acid.

Enzyme	Activity[1]	
	No HCl	+HCl
Invertase	14	1.2
Alkaline Phosphatase	132	121
Tyrosinase	84	79
L-amino acid oxidase	29	0.4

[1] Arbitrary units

An induced mycelium of 69-1113a was exposed to 0.1N HCl for 1 minute @ 0°C. The acid was neutralized and the enzymes extracted. Invertase was assayed by the method of Metzenberg (75) and the glucose produced was measured with glucostat reagent (Worthington Biochemicals). Alkaline phosphatase was assayed by the method of Torriani (76) as modified by Metzenberg (75).

Since NADase was not inactivated by the treatment with acid in extracts, this test cannot be applied to determine its localization in the mycelium. Zalokar and Cochrane (28) found it could be washed out of conidia, which, with other data, suggested to them that NADase is located outside the cell membrane of conidia.

DISCUSSION

A. THE CONTROL OF ENZYME INDUCTION

During the past few years, Dr. Horowitz has developed the following model for the control of tyrosinase synthesis. During the growth of wild type cultures, a rapidly turning over repressor which prevents the synthesis of tyrosinase is formed. When the culture is washed free of medium and starved in phosphate buffer, the steady-state concentration of the repressor falls due to a decreased rate of synthesis. Eventually, it drops below a critical level, and synthesis of tyrosinase starts. The lag period is interpreted as the period during which the repressor concentration is falling to the level which permits the start of tyrosinase synthesis.

The mutants ty-1 and ty-2 are strains in which the lag period is prolonged indefinitely. Since they are recessive to the wild type in heterocaryons, they obviously lack something the wild type can make. It has been proposed that they are deficient in their ability to destroy repressor. This would lead to the accumulation of very high repressor levels. When the cultures are induced in phosphate buffer, the steady-state level of repressor does not fall below the level which permits tyrosinase synthesis. However, these strains are capable of being induced, and therefore must be capable of destroying preformed repressor even if at a decreased rate. It is proposed that inducers act

by interfering with the synthesis of the repressor. Under these circumstances of reduced synthesis, the mutants are capable of decreasing the repressor level to the concentration which permits tyrosinase synthesis. Since the rate of destruction is decreased in ty-1 and ty-2, their lag periods are prolonged relative to wild type.

Several additional experiments indicate that ty-1 can break down repressor. When wild type strains are grown on a medium containing only one-tenth the normal amount of sulfur(103), they make tyrosinase while growing, according to the model because they are making less repressor. If ty-1 is grown on low sulfur, it does not make tyrosinase, but ty-1 grown on low sulfur does form the enzyme when starved in phosphate buffer (94). The model says that because of the reduced rate of repressor synthesis in low sulfur medium, the level of repressor is low enough so that during starvation in buffer, ty-1 can destroy enough repressor to permit synthesis of tyrosinase. A second experiment suggesting that ty-1 can destroy the repressor was reported in section II,Y. Cultures of ty-1 grown on normal medium were washed with water and placed in phosphate. To one set an inducer was added and the time course was determined. The second set was allowed to starve in buffer for $4\frac{1}{2}$ days and then the same inducer was added to the buffer. The lag period was greatly decreased in the set which had starved for $4\frac{1}{2}$ days before induction, indicating

that the amount of repressor in those cultures was less than in the other set. The model also explains the need for inducers when the wild type is induced in Vogel's basal salts. Under these conditions the rate of synthesis is not decreased sufficiently relative to the rate of destruction to permit completion of the lag period in the normal time. Thus this simple model depending on the inhibition of enzyme synthesis by a repressor can account for all the known facts of tyrosinase induction.

Can the control of NADase and/or L-amino acid oxidase induction be fitted into this control system in some simple manner?

NADase is not made by wild type cultures while actively growing and is induced by starvation in phosphate buffer like tyrosinase. It was not made by ty-2 during starvation in phosphate buffer while the results with ty-1 are ambiguous due to the presence of an inhibitor in the cultures. While all these results suggest that NADase is under the same control as tyrosinase, the evidence taken as a whole suggests that NADase induction is not under the same control system as tyrosinase. First, NADase is present in very high concentrations in ungerminated conidia, whereas no tyrosinase can be detected in these cells (28, 87). Second, its induction is inhibited very strongly by certain amino acids which do not inhibit the induction of tyrosinase in those same cells. Third, after the EDTA treatment, the induction of

NADase is not restored fully by the conditions which fully restore tyrosinase synthesis. Last, two independently derived strains of Neurospora were found which made NADase but not tyrosinase while growing. While these data suggest strongly that the control of NADase synthesis is separate from that of tyrosinase, they are insufficient to propose a model for its control. However, it is obvious that the synthesis is turned off in germinating wild type conidia or else all strains would contain high concentrations of NAD ase at two days of growth. Some further experiments which would be of interest are (1) tests of the effects of Turian's mycelial and conidial media (85) on NADase synthesis in P110A and pe,fl;cot;(2) attempts to induce ty-1 and ty-2 to make NADase with inducers other than DL-ethionine and D-phenylalanine; (3) studies of P110A and pe,fl;cot in heterocaryons in order to determine whether the synthesis of NADase during growth is a dominant or recessive character; (4) mapping of the constitutive locus; (5) selection of NADase mutants.

The control of L-amino acid oxidase induction appears to be much more closely connected to that of tyrosinase induction (13). Neither enzyme is made by growing wild type Neurospora in two days. Both are made during starvation of wild type in phosphate buffer in a parallel manner. Neither is made during phosphate starvation of either ty-1 or ty-2 unless an inducer is added to the buffer. After

treatment with EDTA, the two enzymes are restored maximally by the same concentrations of calcium, copper and amino acids. It may be a significant difference between the control of the two enzymes that tyrosinase but not L-amino acid oxidase is also restored maximally by high calcium and amino acids. However, strain P110A makes L-amino acid oxidase while growing but does not make tyrosinase under those conditions. Yet P110A can make tyrosinase when induced with ethionine or D-phenylalanine. This strain proves that the control of the two enzymes is divisible. Yet it is obvious that generally conditions which induce tyrosinase will also induce L-amino acid oxidase and that the mutants ty-1 and ty-2 affect the control of induction of both enzymes. Other methods of separating the induction of these two enzymes have been found and are currently under study in this laboratory.

Horowitz suggested that perhaps these two enzymes, tyrosinase and L-amino acid oxidase, were part of a single operon of genes involved in sexual differentiation (13). In such an operon, the order would be expected to be operator, tyrosinase, oxidase, since more tyrosinase molecules are made than L-amino acid oxidase molecules during normal induction. However, if we were to assign to P110A the role of a polarity mutant, then the order would be operator, oxidase, tyrosinase, and the mutation would be read as sense in the presence

of inducers in order that tyrosinase could be made. These properties rule out the possibility that these two enzymes are part of the same operon in P110A.

Any model explaining the related control of tyrosinase and L-amino acid oxidase must contain a point of duality in it. One such model is the one-repressor-many-operons model of Maas (25), described in the Introduction. In such a model, all breakdowns in unitary control are ascribed to differences in the operator regions of the two genes. Thus P110A would be postulated to have an altered operator in the L-amino acid oxidase operator, so that the repressor does not repress synthesis of the enzyme during growth. However, P110A also makes NADase during growth, and any explanation must account for its pleiotropic effects. Any other differences in control between the two enzymes would be attributed to differences in the effect the conditions had upon the binding of the repressor to the operator region of each gene.

Another model might be called the two-repressor-one-destruction model. This model postulates that there is a specific tyrosinase repressor and a specific L-amino acid oxidase repressor. These two different molecules are part of a population of molecules which are broken down by a mechanism specific to certain repressors and a second non-specific mechanism. Such separation of molecules

from the general turnover population is known in bacteria where the induced enzymes do not seem to turn over during starvation (95). The specific mechanism is postulated to be missing in the mutants ty-1 and ty-2. Different reactions to different conditions of induction could be traced to two causes: (1) different effects on the binding of a particular repressor to its operator or (2) different effects upon the synthesis of the two-repressor molecules. It has been shown many times that different metabolic states can affect the synthesis of different molecules differently, e.g. the induction of NADase was inhibited by amino acids while tyrosinase induction was not affected. In this model, P110A could, as in the previous case, be some non-specific mutation causing the synthesis of L-amino acid oxidase during growth as one of many pleiotropic effects. Or P110A could produce a defective oxidase repressor or its operator region could be defective, as outlined for the previous model.

Many of the questions concerning the nature of P110A may be cleared up by studying two other mutants sent to us by Dr. Woodward, which he finds are alleles of P110A by testing in heterocaryons. It will be interesting to see whether they also produce both enzymes while growing.

B. THE EFFECTS OF EDTA ON NEUROSPORA

The induction of all three enzymes was inhibited by washing the mycelium with EDTA. The induction of tyrosinase and L-amino acid oxidase was fully restored when calcium, copper, and amino acids were added to the induction buffer. EDTA inhibition of induction is due to two separate phenomena. First, some EDTA remains in the mycelium after the two water rinses. Second, the EDTA apparently removes some calcium from the mycelium.

When calcium and amino acids were present in the induction buffer, the additions of either copper or nickel greatly increased the amount of tyrosinase made. Since nickel has no known biological function, this experiment suggests that some EDTA has remained in the mycelium and that these two cations act by combining with it. Such a role for copper is consistent with the facts that no detectable amount of copper was removed by EDTA from the mycelium and that the induction of L-amino acid oxidase which contains no copper (53) was restored by the same concentration of copper as tyrosinase. If copper and nickel do act by complexing with the EDTA remaining in the cells, then other ions bound by EDTA should be effective in restoring induction, for example, iron(III), zinc(II), or cobalt(II). Also, any combination of these cations should be as effective as any single cation at the same final concentrations.

That complete restoration of synthesis does not occur when copper and amino acids are present alone, but only upon addition of calcium, suggests that some essential calcium was removed from the mycelium during the EDTA treatment. If the concentration of free calcium can be increased sufficiently, then induction ought to proceed in the absence of other cations unless the EDTA in the mycelium is binding an essential cation whose formation constant is much greater than that of calcium. It was found that calcium in the presence of amino acids could completely restore induction if its concentration were greater than 10^{-4}M. Part of the calcium presumably combines with all the EDTA remaining in the mycelium. The rest replaces the calcium removed by EDTA. EDTA left in the mycelium is evidently combined with a cation which binds EDTA no more strongly than calcium.

At pH 6 the formation constant of the copper-EDTA complex is 10^{14} (78). Thus, essentially all the EDTA and copper in an equimolar solution will be tied up in the complex. If we assume that the only role of copper or nickel in restoring induction is to bind EDTA, then the concentration of copper giving maximal restoration of tyrosinase induction is a measure of the concentration of EDTA in the mycelium, about 10^{-4}M by this estimate. Under the model being developed, all the calcium added in the presence of copper remains

free to replace the calcium removed by EDTA. It also assumes that any calcium in excess of that removed will inhibit induction. Therefore, the concentration of calcium giving maximal restoration in the presence of copper and amino acids is an estimate of the concentration of calcium necessary for induction. It is a minimal estimate, since the EDTA in the mycelium probably binds some calcium, and that is freed by the copper added. This estimate is about 5×10^{-5}M. One can predict that the concentration of $CaCl_2$ giving full restoration of tyrosinase induction without added Cu should be about 2×10^{-4}M in the presence of amino acids, the sum of the two other estimates. This is essentially the concentration of calcium found experimentally to restore tyrosinase induction.

Amino acids have a dramatic effect upon the amount of tyrosinase made after EDTA treatment. Thus the amount of tyrosinase made in the presence of 3×10^{-4}M $CaCl_2$ was increased ten-fold by the addition of amino acids (cf. table 38). They do not restore any induction by themselves because they cannot complex EDTA. They probably act by replacing the amino acids lost to the wash solutions and buffer. EDTA treated cultures in the presence of low calcium with or without copper did not take up exogenous amino acids. Therefore, once an amino acid leaks out of EDTA-treated cells, it is lost to the mycelium forever. This would cause the availability of amino

acids to limit the rate of protein synthesis. The high external concentration of amino acids would oppose this loss and permit a faster rate of protein synthesis. When the concentration of added calcium was increased to a level permitting the uptake of exogenous amino acids, the added amino acids no longer increased the yield of tyrosinase (cf. section II R). Only a small amount of tyrosinase was made under these conditions due to the inhibition of tyrosinase by high concentrations of calcium. Thus it is suggested that EDTA-treated cultures respond to added amino acids for the same reasons that cells in tissue culture require certain amino acids: to insure an adequate concentration of amino acids for protein synthesis (80).

After 48 hours, cultures which were not treated with EDTA had lost almost the same amount of amino acids to the buffer as EDTA-treated cultures (cf. table 36). Yet the amount of tyrosinase made by water-washed cultures was not increased by added amino acids. Why? Untreated cultures differ from EDTA-treated cultures in two important respects: (1) At any given time during the course of induction they have lost less material, since a major loss of intracellular substances occurs during the EDTA wash; (2) They retain the ability to take up exogenous amino acids throughout the course of induction (29). Therefore, untreated cells are less likely to need added amino acids and are able to recover any amino acid which leaks

into the buffer by transporting it back into the cell.

EDTA has four known effects upon inducing cultures of Neurospora. First, it inhibits the induction of tyrosinase, L-amino acid oxidase, and NADase, probably by inhibiting their de novo synthesis. Conditions have been found which completely reverse the inhibition of tyrosinase and L-amino acid oxidase induction. Second, it increases the permeability of the mycelium. Thus, actinomycin D is a much more effective inhibitor of induction in EDTA-treated cells, presumably because of increased permeability of the cells to the antibiotic. The EDTA-treated cells also lose a large amount of 260 mμ absorbing material to the wash solutions because of this increased permeability. Leive (68, 86) has reported that an EDTA-treatment also increases the permeability of E. coli cells. Third, the EDTA treatment completely inhibits the uptake of exogenous amino acids from the induction buffer by active transport. Fourth, EDTA-treated cultures do not incorporate amino acids into protein. Any of these effects could be responsible for the lack of tyrosinase induction in these cultures. As yet, it is not known whether the last three phenomenologically separate effects of EDTA are causally related or separate.

Leive showed that when E. coli is washed with 2×10^{-4} M EDTA, the cells become permeable to a host of different compounds,

including actinomycin D and carbamyl phosphate, to which they are normally impermeable (86). This low concentration of EDTA did not inhibit growth, protein synthesis, or RNA synthesis. When 10^{-3}M EDTA was used to wash the cells, Leive found that the increase in permeability was accompanied by a slight inhibition of growth, protein synthesis, and RNA synthesis (68). My EDTA treatment completely inhibited active transport of valine into the cell, and its incorporation into protein, in addition to increasing the mycelial permeability. These differences between Leive's work and my own probably are due to the differences in the systems used: (1) Neurospora crassa vs. E. coli; (2) starving cultures vs. growing cells; (3) 0.5% EDTA (0.013M) vs. 2×10^{-4}M EDTA; and (4) the duration of the EDTA treatment--30-60 minutes vs. 2 minutes. It may be that in my system the increase in permeability causes the inhibition of induction. The fact that calcium but not magnesium plays an important role in membranes (89) and in the restoration of induction suggests that some effect on membranes is involved. Calcium stabilizes cell membranes, opposes depolarization, and its absence causes a fall in the membrane potential of many cells (89). If the permeability increase is not the cause of the inhibition of induction, then it may be possible to separate these two effects of EDTA. Leive separated the permeability increase and the inhibition of growth in

E. coli by lowering the EDTA concentration (86). This suggests that I should try lower EDTA concentrations and decreased lengths of the treatment to separate the two phenomena.

If the permeability increase per se is not the cause of the inhibition of induction, an alternative explanation would be a general inhibition of metabolism which is restored by calcium. This explanation is supported by the inhibition of protein synthesis reported, although no correlation between treatments giving restoration of induction and the restoration of synthesis was observed (cf. section IIQ) These experiments should be extended using amino acids of sufficient activity to make conclusions on the effects of different treatments on restoration of protein synthesis significant and to allow mixtures of other amino acids to be present in addition to the labelled amino acid. Experiments after the completion of the lag period when the enzymes involved are actually being synthesized would be of interest. This hypothetical inhibition of metabolism could be measured on mycelia treated with EDTA to see whether or not restoration of metabolism and induction are well correlated.

C. THE PROCESS OF INDUCTION

What has been learned about the process of induction in the course of these experiments with EDTA? The single most important result is that the lag phase can be completed under conditions which do

not permit the synthesis of tyrosinase. When cultures treated with EDTA were placed in phosphate buffer containing only 5×10^{-5}M $CaCl_2$, they completed the lag phase of induction but made no tyrosinase unless copper and amino acids were added. (cf. section II V). This finding reinforces the conclusion that the lag phase is an important separate step in the process of induction. Second, the mycelium cannot complete the lag phase in the absence of oxygen, suggesting that the lag phase requires energy. Third, the lag phase is the same length after EDTA treatment, if induction is restored immediately, as in untreated cultures. However, when EDTA-treated cultures are starved in buffer in the absence of cations, the lag phase is not completed, and when cations and amino acids are added later in the course of induction, the subsequent lag phase is greatly lengthened. Fourth, if cultures are allowed to complete the lag phase and then are treated with EDTA, synthesis is inhibited and the same conditions restore synthesis as restore total induction. Last, the conditions which restore the induction of tyrosinase and L-amino acid oxidase completely do not fully restore NADase induction.

D. THE KINETICS OF INDUCTION

An interesting observation in the course of these experiments is that the time course of induction often has an early phase when the amount of tyrosinase activity increases exponentially with time

(cf. figures 4,14,15,17). Such kinetics of synthesis suggest an autocatalytic mechanism, i.e. the enzyme already made catalyzes the production of more enzyme. Since the induction of L-amino acid oxidase parallels that of tyrosinase, it also has an exponential phase of synthesis. Several possible alternatives suggest themselves as explanations. First, tyrosinase may be linked to an energy-producing mechanism, which is used to supply energy to synthesize the induced enzymes. If this were true, then induction in the presence of catechol, which inactivates Neurospora tyrosinase extremely rapidly (96), would inhibit the induction of L-amino acid oxidase, since active tyrosinase would not exist in the mycelium. Second, L-amino acid oxidase, which contains FAD, might be providing energy. Thus the FAD in succinic dehydrogenase feeds electrons directly into the pathway of oxidative phosphorylation. It is conceivable that the FAD of L-amino acid oxidase could yield energy for its own synthesis and that of tyrosinase. Since no specific inhibitors of L-amino acid oxidase with the effectiveness of catechol for tyrosinase are known, this hypothesis is harder to test. A third possibility is that the ammonium ion or α-keto acids produced by L-amino acid oxidase limit the rate of synthesis of these enzymes, so that the amount of L-amino acid oxidase by increasing the amount of these products would increase the rate at which the enzyme was made. If

one of these products is the limiting factor in the induction of these enzymes, then its addition to the medium ought to change the kinetics from exponential to linear. A fourth possibility is that an unknown induced enzyme produces a product which is limiting to the rate of synthesis. One example might be a protease which supplies the amino acids used in synthesis. In this example the addition of a mixture of amino acids to the induction buffer should make the kinetics linear instead of exponential. A fifth possibility is that one of the induced enzymes can destroy the repressor(s). If the rate of synthesis were proportional to the amount of repressor present, then as the amount of this enzyme increased the rate of synthesis would increase, resulting in an exponential rate of synthesis which would end when all the repressor was destroyed. Since enzyme synthesis requires oxygen, such a mechanism of repressor destruction would explain the need for oxygen during the lag phase. A last possibility is that the exponential phase is an artifact having no significance, but further study of this phenomenon is justified by the possibilities outlined above.

APPENDIX 1. PROPERTIES OF THE ENZYMES FROM NEUROSPORA

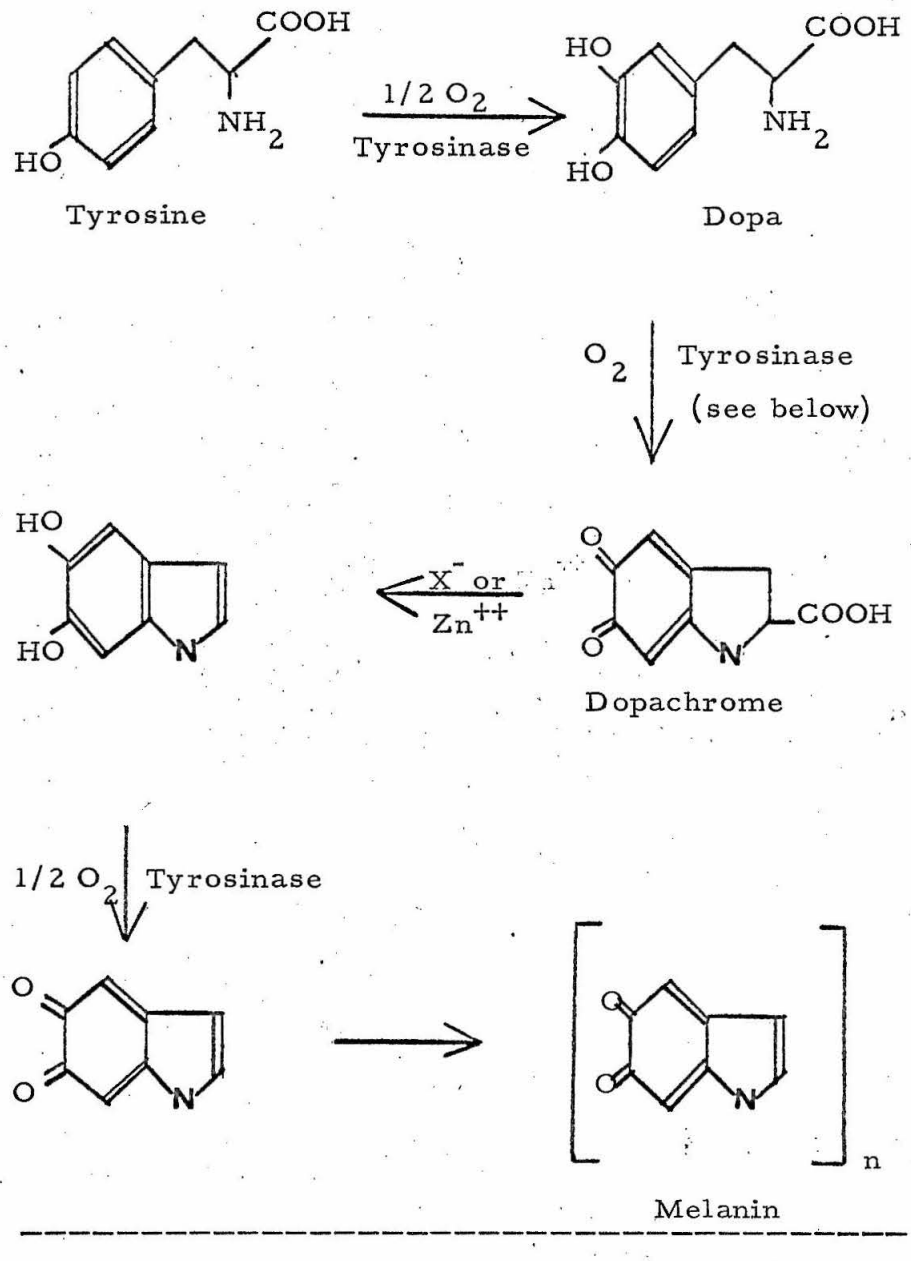
A.

Tyrosinase

O-Diphenol: O_2 oxidoreductase (E.C. 1.10.3.1.)[84]

Tyrosinase catalyzes the conversion of tyrosine to melanin by the reactions shown in Figure 19 (35). Like other phenol oxidases, it is destroyed during the reaction. The enzyme exists in four different molecular forms in nature. These forms differ either in thermostability or electrophoretic mobility or both (30, 32). The enzyme is inhibited by cyanide, azide, phenylthiourea, diethyldithiocarbamate, and cysteine, but not by EDTA (.01M)(96, 102). The thermostable S form and thermolabile L form of the enzyme have both been crystallized as pure proteins. The enzyme has a molecular weight of 32,000 ± 2,000 and contains 1 atom of cuprous copper per molecule. Unlike other phenol oxidases (58,59,60), this copper atom cannot be removed reversibly from the molecule. The enzyme aggregates reversibly. The molecular activity of the enzyme is 19,000 moles of L-DOPA per minute at 30°C.

Figure 19. The reactions catalyzed by tyrosinase.



$$2\ \text{Dopa} + O_2 \rightarrow 2\ \text{dopaquinone} + 2\ H_2O$$

Dopaquinone → leucodopachrome

Leucodopachrome + dopaquinone → dopachrome + dopa

Sum: $\text{Dopa} + O_2 \rightarrow \text{dopachrome} + H_2O$

B.

NADase or DPNase

Nicotinamide adenine dinucleotide glycohydrase (E.C.3.2.2.5)[84]

The enzyme NADase hydrolyzes the glycosidic bond between nicotinamide and ribose in NAD as shown in Figure 20 (38). The enzyme also cleaves NADP but does not attack $NADH_2$ or $NADPH_2$ or the half-reduced compounds. It also does not cleave nicotinamide mononucleotide, nicotinamide mononucleoside, nor deamino NAD. The enzyme has a very broad pH optimum from 3 to 9. Its activity is insensitive to metal ions, including heavy metals, and to fluoride, cysteine, EDTA, and cyanide. NADase is destroyed by heating 2 minutes at 80°C but not at 55°C between pH 3 and 5. The enzyme is not denatured by trichloroacetic acid. Nason et al. (27) found the enzyme in high concentrations in cultures deprived of zinc and/or biotin, and the enzyme was present in the medium. Zalokar and Cochrane reported that NADase was present in the highest concentration in conidia and could be washed out of them with water (28).

C.

L-Amino Acid Oxidase

L-Amino acid: O_2 oxidoreductase (deaminating) (E.C.1.4.3.2)[84]

The L-amino acid oxidase of Neurospora crassa catalyzes the deamination of many L-α-amino acids by the reaction shown in

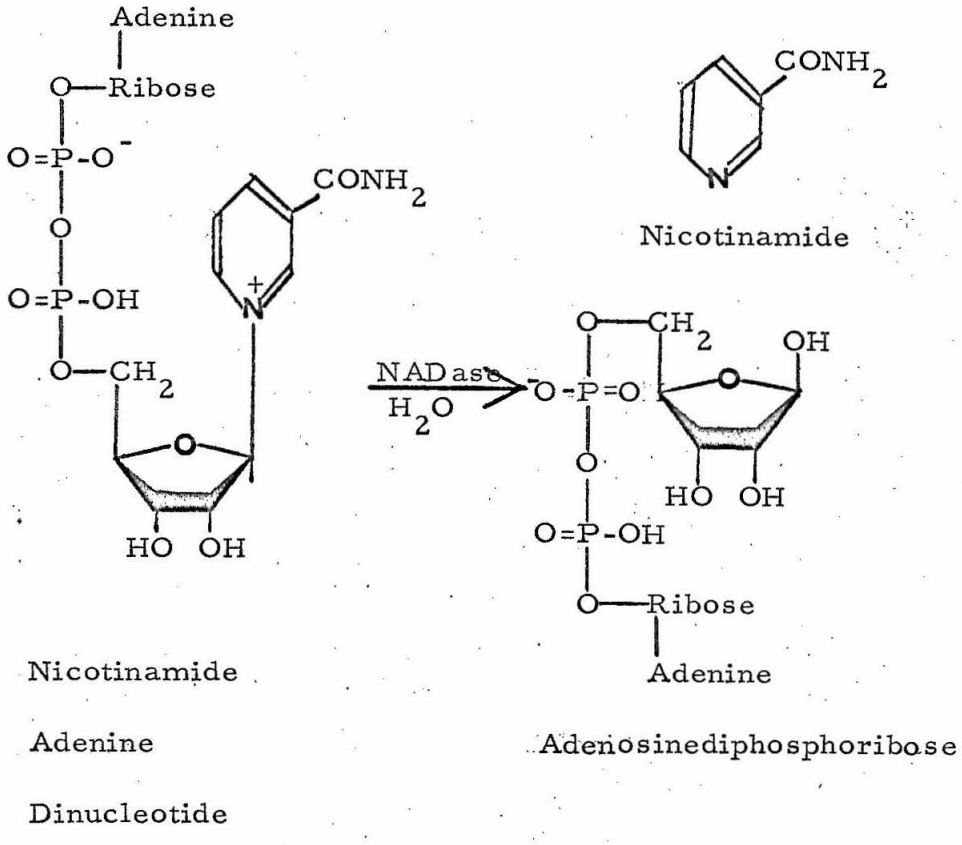


Figure 20.
The reaction catalyzed by NADase

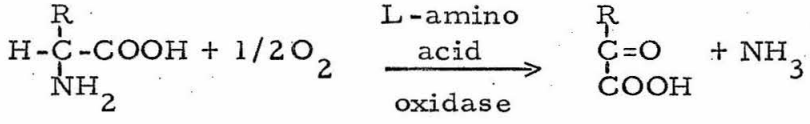


Figure 21.
The reaction catalyzed by L-amino acid oxidase

Figure 21(62, 53). The enzyme has a broad pH optimum 6 to 9.5, with 70% of maximal activity at pH 4. Molecular oxygen can be replaced as hydrogen acceptor by ferricyanide or reducible dyes. The enzyme has a temperature optimum at 45°C. The enzyme is not inhibited by the D-enantiomer of the substrate, azide, cyanide, hydroxylamine, iodoacetate, chelating agents or ammonium ion. It was inhibited by atebrine, crystal violet and copper. Burton (53) showed that the enzyme contains FAD and that the turnover number is 2100 mol. of L-phenylalanine per mol. FAD per minute. at 30°C. Thayer and Horowitz (52) showed that when Neurospora was grown on low biotin and/or amino acids as sole nitrogen source, the enzyme was formed adaptively.

REFERENCES

1. Ingram, Vernon M. The Hemoglobins in Genetics and Evolution, Columbia University Press, New York (1963).

2. Schroeder, W. A. et al. Biochemistry 2, 992 (1962).

3. Cook, C. D. et al. Pediatrics, 20, 272 (1957).

4. Gurdon, John. Quart. Rev. Biol., 38, 54 (1963).

5. Jacob, F. and J. Monod. Cold Spring Harbor Symp. Quant. Biol., 26, 193 (1961).

6. Ames, B. N., B. Garry, and L. A. Herzenberg. J. Gen. Microbiol., 22, 369 (1960).

7. Attardi, G. et al. Cold Spring Harbor Symp. Quant. Biol., 28, 363 (1963).

8. Guttman, B. and A. Novick. Cold Spring Harbor Symp. Quant. Biol., 28, 373 (1963).

9. Hayashi, M. S., S. Spoegelman, N. C. Franklin, and S. E. Luria, Proc. Nat. Acad. Sci. U.S., 49, 729 (1963).

10. Ahmed, A., M. Case, and N. H. Giles, in Subunit Structure of Proteins, Brookhaven Natl. Lab. Symp. (1964), p. 53.

11. Catcheside, D. Biochem. Biophys. Res. Comm., 18 (1965).

12. Douglas, H. C. and D. C. Hawthorne, Genetics, 49, 837 (1964).

13. Horowitz, N. H. Biochem. Biophys. Res. Comm., 18, 686 (1965).

14. Fick, G. R. Science, 146, 525 (1964).

15. Ames, B. N. and P. E. Hartman. Cold Spring Harbor Symp. Quant. Biol., 28, 349 (1963).

16. Fincham, J.R.S. and P. R. Day, Fungal Genetics, Blackwell Scientific Publ. (1963), p. 99.

17. Hawthorne, D. C. and R. K. Mortimer, Genetics, 45, 1085 (1960).

18. Horowitz, N. H., M. Fling, H. Macleod, and N. Sueoka, J. Mol. Biol., 2, 96 (1960).

19. Lewis, E. B., in The Role of Chromosomes in Development, ed. M. Locke, Academic Press, New York (1964), p. 231.

20. Vogel, H. J., D. F. Bacon, and A. Baich, in Informational Macromolecules, eds. H. J. Vogel, J. Bryson, and J. O. Lampen, Academic Press, New York (1963), pp. 293-301.

21. Gorini, L., W. Gunderson, and M. Burger. Cold Spring Harbor Symp. Quant. Biol., 26, 173 (1961).

22. Maas, W. K. Cold Spring Harbor Symp. Quant. Biol., 26, 183 (1961).

23. Vogel, H. J. Cold Spring Harbor Symp. Quant. Biol., 26, 163 (1961).

24. Maas, W. K., R. Maas, J. Wiane, and N. Glansdorff, J. Mol. Biol., 8, 359 (1964).

25. Maas, W. K. and A. J. Clark, J. Mol. Biol., 8, 365 (1964).

26. Ames, B. N. and B. Garry. Proc. Nat. Acad. Sci. U.S., 45, 1453 (1959).

27. Nason, A., N. O. Kaplan, and S. P. Colowick. J. Biol. Chem., 188, 397 (1951).

28. Zalokar, M. and V. W. Cochrane. Am. J. Bot., 43, 107 (1956).

29. Fling, M., personal communication.

30. Horowitz, N. H., M. Fling, H. Macleod, and N. Sueoka, Genetics, 46, 1015 (1961).

31. Horowitz, N. H., M. Fling, H. Macleod, and Y. Watanabe. Cold Spring Harbor Symp. Quant. Biol., 26, 233 (1961).

32. Horowitz, N. H. and M. Fling. Genetics, 38, 360 (1953).

33. Mitchell, M. B., personal communication.

34. Vogel, H. J. Microbial Genetics Bull., 13, 42 (1956).

35. Fling, M., N. H. Horowitz, and S. Heineman, J. Biol. Chem., 238, 2045 (1963).

36. Jervis, G. A. Proc. Soc. Exptl. Med., 81, 715 (1952).

37. Lin, E.C.C., B. M. Pitt, M. Civen, and W. E. Knox. J. Biol. Chem., 233, 668 (1958).

38. Kaplan, N. O., S. P. Colowick, and A. Nason. J. Biol. Chem., 191, 473 (1951).

39. Colowick, S. P., N. O. Kaplan, and M. M. Ciotti. J. Biol. Chem., 191, 447 (1951).

40. Roberts, R. B. et al. Studies of Biosynthesis in Escherichia coli, Carnegie Institution of Washington Publn. 607, Washington (1955).

41. Nathans, D. Proc. Nat. Acad. Sci. U.S., 51, 585 (1964).

42. Horowitz, N. H., unpublished data.

43. Hurwitz, J., F. S. Furth, M. Malamy, and M. Alexander. Proc. Nat. Acad. Sci. U.S., 48, 1222 (1962).

44. Goldberg, I. H. and M. Rabinowitz. Science, 136, 315 (1962).

45. Levinthal, C., A. Keynan, and A. Higa. Proc. Nat. Acad. Sci. U.S., 48, 1631 (1962).

46. Kersten, W., H. Kersten, and H. M. Ranen. Nature, 187, 60 (1960).

47. Morris, A., R. Arlinghaus, S. Flavelukes, and R. Schweet. Biochemistry, 2, 1084 (1963).

48. Rabinowitz, M. and J. M. Fisher. J. Biol. Chem., 237, 477 (1962).

49. Allen, D. W. and P. C. Zamecnik. Biochem. Biophys. Acta, 55, 865 (1962).

50. Maxwell, J., unpublished data.

51. Gest, H. and N. H. Horowitz. J. Gen. Microbiol., 18, 64 (1958).

52. Thayer, P. S. and N. H. Horowitz. J. Biol. Chem., 192, 755 (1951).

53. Burton, K. Biochem. J., 50, 298 (1951).

54. Brown, D. D. and J. Monod. Fed. Proc., 20, 222 (1961).

55. Brown, D. D., in Discussion of B. Magasanik. Cold Spring Harbor Symp. Quant. Biol., 26, 249 (1961).

56. Magasanik, B. Cold Spring Harbor Symp. Quant. Biol., 26, 249 (1961).

57. Karkhanis, Y. and E. Frieden, Biochem. Biophys. Res. Comm., 4, 303 (1961).

58. Kubowitz, F. Biochem. Z., 292, 221 (1937).

59. Lerner, A. B., T. B. Fitzpatrick, E. Calkins, and W. H. Summerson. J. Biol. Chem., 187, 793 (1950).

60. Kertesz, D. Biochem. Biophys. Acta, 9, 170 (1952).

61. Spies, J. R., in Methods in Enzymology, Vol. III, eds. S. P. Colowick and N. O. Kaplan, Academic Press, New York, (1957), p. 468.

62. Bender, A. E. and H. A. Krebs. Biochem. J. 46 210 (1950).

63. Scott, W., personal communication.

64. Zalokar, M. Arch. Biochem. Biophys., 50, 71 (1954).

65. St. Lawrence, P., B. D. Maling, L. Altwerger, and M. Rachmeler. Genetics, 50, 1383 (1964).

66. Zalokar, M. Exptl. Cell Res., 19, 114 (1960).

67. Fischer, E. H. and E. A. Stein, "α-Amylases" in The Enzyme, 2nd ed., eds. P. D. Boyer, H. Lardy, and K. Myrbäck, Academic Press, New York (1960), pp. 317-345.

68. Leive, L. Biochem. Biophys. Res. Comm., 18, 13 (1965).

69. Mandelstam, J. Bacteriol. Revs., 24, 289 (1960).

70. Steinberg, D., M. Vaughan, and C. B. Anfinsen. Science, 124, 389 (1956).

71. Simpson, M. V. J. Biol. Chem., 201, 143 (1953).

72. Olson, B. H. and H. J. Johnson, J. Bact., 57, 235 (1949).

73. Heinemann, S., unpublished results.

74. Metzenberg, R. L. Biochim. Biophys. Acta, 77, 455 (1963).

75. Metzenberg, R. L. Arch. Biochem. Biophys., 96, 468 (1962).

76. Torriani, A. Biochim. Biophys. Acta, 38, 460 (1960).

77. Chance, B., in Energy-Linked Functions of Mitochondria, ed. B. Chance, Academic Press, New York (1963), p. 253.

78. Laitenen, H. A. Chemical Analysis, McGraw-Hill, New York (1960), pp. 228-31.

79. Schimke, R. T. Cold Spring Harbor Symp. Quant. Biol., 26, 363 (1961).

80. Eagle, H. and K. Piez. J. Exptl. Med., 116, 29 (1962).

81. Cox, R. P. and C. M. Macleod. Cold Spring Harbor Symp. Quant. Biol., 26, 233 (1964).

82. Garen, A. and H. Echols. Proc. Nat. Acad. Sci. U.S., 48, 1398 (1962).

83. Hirsch, H. M. Physiologia Plantarum, 7, 72 (1954).

84. Report of the Commission on Enzymes, I. U.B. Symp., vol. 20 (1961).

85. Turian, G. Nature, 202, 1240 (1964).

86. Leive, L. Proc. Nat. Acad. Sci. U.S., 53, 745 (1965).

87. Macleod, H., personal communication.

88. Nicholas, D. J. D. Analyst, 77, 629 (1952).

89. Shanes, A. M. Pharmocol. Rev., 10, 59 (1958).

90. Westergaard, M. and H. K. Mitchell. Am. J. Bot. 34, 573 (1947).

91. Comar, C. L. and R. H. Wasserman in Mineral Metabolism vol. II A, eds. C. L. Comar and F. Bronner, Academic Press, New York (1964) p. 523.

92. Chao, F.-C. Arch. Biochem. Biophys. 70, 426 (1957).

93. Zillig, W., W. Krone and M. Albers. Z. Physiol. Chem. 317, 131 (1959).

94. Watanabe, Y., unpublished results.

95. Mandelstam, J. Biochem. J. 69, 110 (1958).

96. Sussman, A. S. Arch. Biochem. Biophys. 95, 407 (1961).

97. Schaeffer, P. Arch. Biochem. Biophys. 47, 359 (1953).

98. Fox, A. S. Nature 173, 350 (1954).

99. Fox, A. S. and J. B. Burnett, Genetics 41, 643 (1956).

100. Fox, A. S. and J. B. Burnett in Pigment Cell Biology ed. M. Gordon, Academic Press, New York (1959) p. 249.

101. Fox, A. S., J. B. Burnett and M. S. Fuchs, Genetics 44, 510 (1959).

102. Fox, A. S. and J. B. Burnett, Biochim. Biophys. Acta 61, 108 (1962).

103. Fox, A. S. and W. D. Gray, Proc. Nat. Acad. Sci. U.S. 36 538 (1950).