PART I

SYNTHESIS OF L-AMINO ACID OXIDASE BY A SERINE- OR GLYCINE-REQUIRING STRAIN OF *NEUROSPORA*

PART II

STUDIES CONCERNING MULTIPLE ELECTROPHORETIC FORMS OF TYROSINASE IN *NEUROSPORA*

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ABSTRACT

Part I: Synthesis of L-Amino Acid Oxidase by a Serine- or Glycine-Requiring Strain of Neurospora

Wild-type cultures of Neurospora crassa growing on minimal medium contain low levels of L-amino acid oxidase, tyrosinase, and nicotinamide adenine dinucleotide glycohydrase (NADase). The enzymes are derepressed by starvation and by a number of other conditions which are inhibitory to growth. L-amino acid oxidase is, in addition, induced by growth on amino acids. A mutant which produces large quantities of both L-amino acid oxidase and NADase when growing on minimal medium was investigated. Constitutive synthesis of L-amino acid oxidase was shown to be inherited as a single gene, called P110, which is separable from constitutive synthesis of NADase. P110 maps near the centromere on linkage group IV.

L-amino acid oxidase produced constitutively by P110 was partially purified and compared to partially purified L-amino acid oxidase produced by derepressed wild-type cultures. The enzymes are identical with respect to thermostability and molecular weight as judged by gel filtration.

The mutant P110 was shown to be an incompletely blocked auxotroph which requires serine or glycine. None of the enzymes involved in the synthesis of serine from 3-phosphoglyceric acid or glyceric acid was found to be deficient in the mutant, however. An investigation of the free intracellular amino acid pools of P110 indicated that the

mutant is deficient in serine, glycine, and alanine, and accumulates threonine and homoserine.

The relationship between the amino acid requirement of P110 and its synthesis of L-amino acid oxidase is discussed.

Part II: Studies Concerning Multiple Electrophoretic Forms of Tyrosinase in Neurospora

Supernumerary bands shown by some crude tyrosinase preparations in paper electrophoresis were investigated. Genetic analysis indicated that the location of the extra bands is determined by the particular T allele present. The presence of supernumerary bands varies with the method used to derepress tyrosinase production, and with the duration of derepression. The extra bands are unstable and may convert to the major electrophoretic band, suggesting that they result from modification of a single protein. Attempts to isolate the supernumerary bands by continuous flow paper electrophoresis or density gradient zonal electrophoresis were unsuccessful.

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INTRODUCTION

Regulation of Tyrosinase, L-Amino Acid Oxidase, and Nicotinamide Adenine Dinucleotide Glycohydrase in *Neurospora crassa*

Young vegetative cultures of *Neurospora crassa* grown in stationary flasks contain basal amounts of tyrosinase, L-amino acid oxidase, or nicotinamide adenine dinucleotide glycohydrase (NADase). Early investigations indicated that derepression of each enzyme was associated with growth-inhibiting conditions. Tyrosinase was first observed in cultures grown on sulfur-deficient medium (1). L-amino acid oxidase was excreted by cultures grown on suboptimal concentrations of biotin (2,3). Zinc deficiency stimulated NADase production (4). A parallelism in the response of these enzymes to adverse cultural conditions was implied by an increased production of NADase (5) and tyrosinase (2,6), as well as L-amino acid oxidase, in cultures grown on low biotin. Similarly, cultures transferred from minimal medium to phosphate buffer produced high levels of all three enzymes (7). Of particular interest was the demonstration that tyrosinase and L-amino acid oxidase are found in cultures which have sexually differentiated (8). Tyrosinase had been implicated in the differentiation of protoperithecia by Hirsch (9), who confirmed Schaeffer's (82) identification of the dark pigment produced by sexually-differentiated cultures as melanin. Environmental conditions which prevented melanin production similarly inhibited protoperithecial formation. These studies were extended by Barbesgaard and Wagner (10), who analyzed the tyrosinase activity in wild-type cultures

which had produced melanin on medium promoting sexual differentiation, and compared it to enzyme activity in female-sterile mutants which do not produce protoperithecia or melanin on this medium. Tyrosinase activity was present in melanized wild-type and absent in the mutants, as predicted. The role of oxidase and NADase in sexual differentiation is not yet clearly understood. L-oxidase may allow the culture to satisfy its nitrogen requirement from amino acids under the nitrogen-poor conditions which promote sexual differentiation. Hirsch concluded that "conditions which prevent the exhaustion of nitrate always affect adversely both the number and the functioning of the protoperithecia but that the detrimental action of nitrate is most marked when the organism has grown on a medium of a definite C/N ratio." The fundamental importance of the C/N ratio in the induction of the sexual cycle in Neurospora was demonstrated by Westergaard and Mitchell (11). In Hirsch's studies, very high concentrations of hydrolyzed casein were inhibitory to the formation of protoperithecia, but amino nitrogen from hydrolyzed casein was much less inhibitory than equivalent amounts of nitrate nitrogen (9). A suggestion that nicotinamide (one of the products of NADase activity) may play a role in the pathway which leads to protoperithecial formation comes from a study by McNelly-Ingle and Frost (12). Cultures which are unable to produce protoperithecia at 30°C on minimal medium can produce them if grown on nicotinamide-supplemented medium.

The observation that tyrosinase and L-oxidase are associated with sexual differentiation, and that they and NADase respond similarly

to certain environmental conditions suggested that the three enzymes might be regulated by a common control mechanism. An investigation of this possibility indicated that the control of NADase activity could be separated from the control of the other two enzymes (7). Tyrosinase and L-amino acid oxidase activities, however, behaved in a manner which was consistent with the hypothesis that the two enzymes were subject to common control (8). The evidence for common control has been presented in detail elsewhere (8), but will be reiterated here:

(1) After transfer of cultures from minimal medium to phosphate buffer, tyrosinase and L-oxidase activities begin to increase after an identical lag period, and thereafter the activities increase and decrease in parallel.

(2) A single-gene mutant, ty-1, does not synthesize either enzyme when transferred to phosphate buffer. Addition of the amino acid analogue, ethionine, to the buffer results in the parallel formation of both enzyme activities.

These properties of the control of tyrosinase and L-oxidase suggested that the structural genes for the two enzymes might be associated in an operon (8).

The operon hypothesis proposed by Jacob, Monod and Pardee (13, 14) has been supported in detail by numerous investigations with bacteria (15,16,17). Evidence for operons in Neurospora and yeasts has been sought by many investigators, but the examples of such units of closely-linked, coordinately-controlled genes have been few. An exception to this general rule in Neurospora is the arom gene cluster.

In this case, five contiguous genes determine five enzymes involved in the synthesis of aromatic amino acids (18). To date, no evidence exists for regulatory elements which control the gene cluster, comparable to the operator locus or regulator gene which control the lactose operon in E. coli. Giles et al. have suggested that the arom cluster may not serve the same function as the operons in bacteria, but may specify five gene activities associated with a single enzyme aggregate, which may be encoded in a single polycistronic messenger RNA. The gene cluster may facilitate the formation of the aggregate which then serves to channel dehydroquinic acid into biosynthetic as opposed to degradative pathways.

The his-3 locus of Neurospora controls the activity of two enzymes, apparently similar to an operon, but genetic evidence is more compatible with the suggestion that his-3 controls the synthesis of a single polypeptide which participates in more than one enzyme activity (19).

A gene which behaves like a dominant regulatory gene for aryl-beta-glucosidase in Neurospora has been described by Mahadevan and Eberhart (20). This gene, gluc-1, depresses the activity of the glucosidase without affecting the physical properties of the enzyme. The mutation is dominant in heterocaryons.

A similar dominant regulatory gene, ga-4, has been found in yeasts by Douglas and Hawthorne (21,22). This gene causes the constitutive synthesis of galactose pathway enzymes, but is unlinked to the

structural genes encoding these enzymes, unlike the dominant constitutive regulatory genes in the E. coli lac operon.

The suggestion that tyrosinase and L-amino acid oxidase might be controlled by an operon was of interest not only as an example of an operon in a eucaryote, but also because the operon postulated would control the activities of two enzymes which were not known to participate in a common biochemical pathway. Rather, this operon would control enzymes involved in a common developmental pathway, a phenomenon which would be of considerable interest to biologists concerned with differentiation and development in higher organisms.

Properties of the Mutant P110

During the course of the investigations of tyrosinase, L-oxidase and NADase, we obtained a mutant in which the normal control of the latter two enzymes appeared to be defective. The mutant was obtained by Dow Woodward during a hunt for mutants lacking glucose-6-phosphate dehydrogenase. Mutants were selected for their poor growth on sucrose minimal medium. The mutant, called P110, showed low dehydrogenase activity under normal reaction conditions, but had normal activity for the enzyme if the concentration of the cofactor NAD was increased (23). Studies in our laboratory indicated that P110 produced a high level of NADase when growing on minimal medium (7). The NADase in extracts being analyzed for dehydrogenase activity apparently degraded the NAD in the reaction mixture, explaining the high NAD requirement. P110 was also found to produce a large amount of L-amino

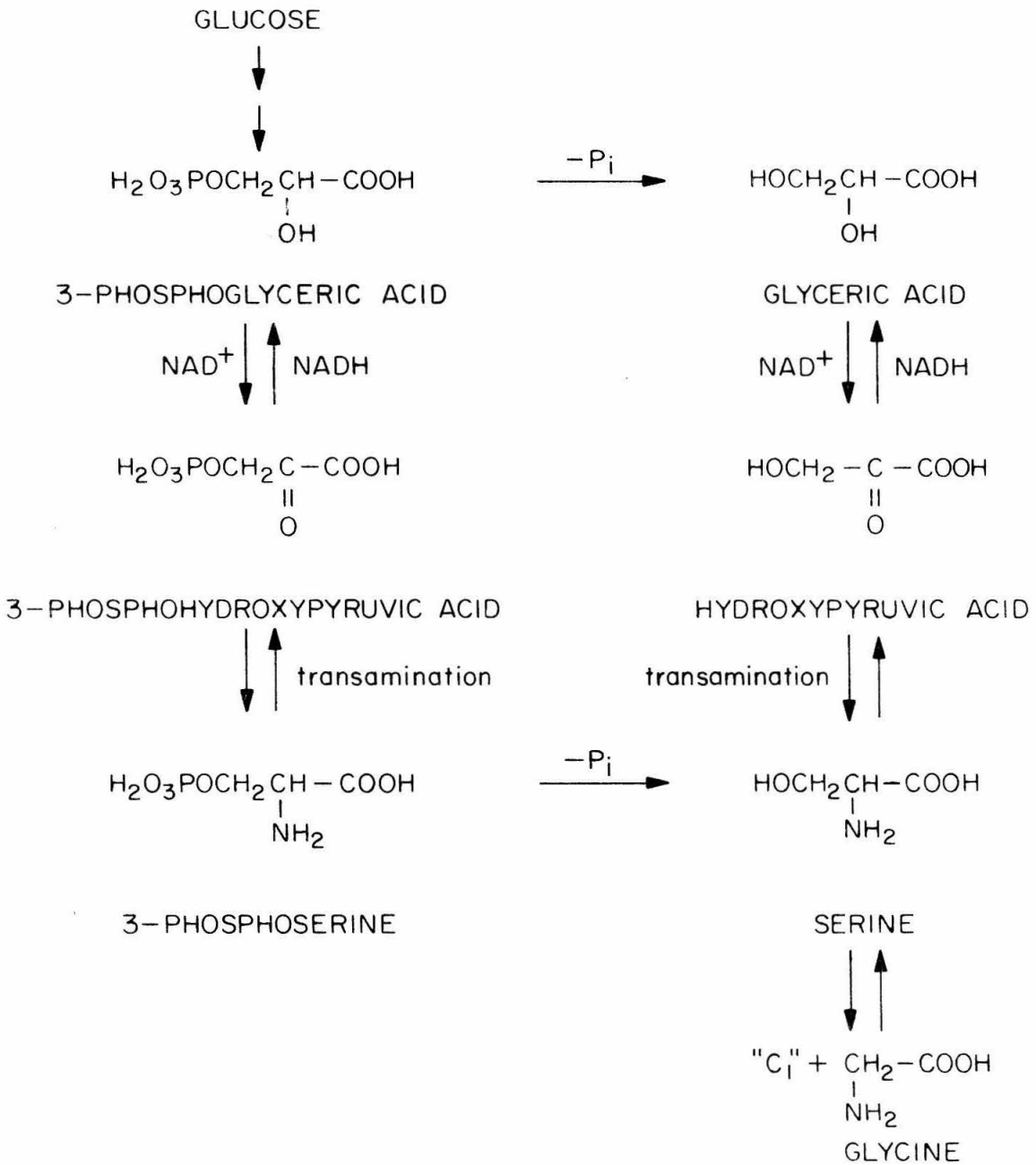
acid oxidase when growing on minimal medium, but the mutant did not synthesize tyrosinase under these conditions (7).

As indicated by the method used for its selection, P110 grows poorly on minimal medium. Studies which will be reported in this thesis indicate that P110 is a "leaky" (24) (incompletely blocked) auxotroph which requires serine or glycine for optimum growth.

Serine Biosynthesis

Synthesis of serine in Neurospora crassa has been studied by Sojka and Garner (25). Enzymes which can perform each of the reactions in the two pathways diagrammed in Figure 1 were demonstrated in crude extracts. The higher activities of the enzymes involved in the phosphorylated pathway compared to the non-phosphorylated pathway suggested that the former pathway is the major biosynthetic pathway in Neurospora. The relative activity of the two pathways appears to be influenced by the carbon source used, although the phosphorylated pathway predominated in both fructose- and glucose-grown cultures (25). The same two pathways for serine biosynthesis were originally demonstrated in animal tissues (26,27), where more recent investigations have shown that the relative contribution by each pathway for the synthesis of serine varies from tissue to tissue (28). Studies using isotopic competition and mutants blocked in serine biosynthesis indicate that the phosphorylated pathway is the only significant source of serine in E. coli and S. typhimurium (29).

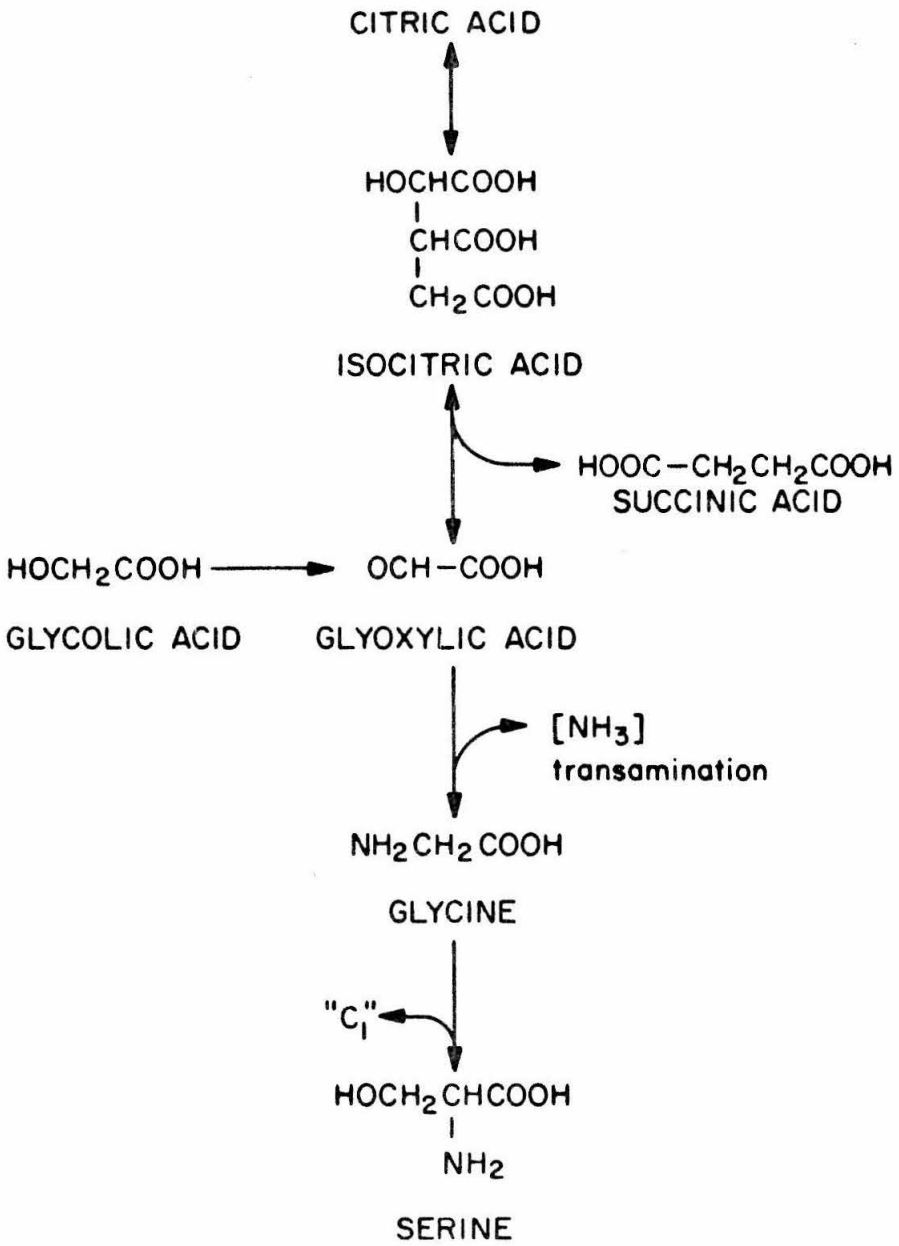
Figure 1. Pathways of serine biosynthesis from products of glycolysis. The "C_1" indicated as a product of serine metabolism refers to the single carbon fragment used in the synthesis of methionine, thymine, and purines.


GLUCOSE
$H_2O_3POCH_2CH-COOH$
OH
$-P_i$
$HOCH_2CH-COOH$
OH
3-PHOSPHOGLYCERIC ACID
GLYCERIC ACID
NAD^+
NADH
NAD^+
NADH
$H_2O_3POCH_2C-COOH$
O
$HOCH_2-C-COOH$
O
3-PHOSPHOHYDROXYPYRUVIC ACID
HYDROXYPYRUVIC ACID
transamination
transamination
$H_2O_3POCH_2CH-COOH$
NH_2
$-P_i$
$HOCH_2CH-COOH$
NH_2
3-PHOSPHOSERINE
SERINE
"C_1" + CH_2-COOH
NH_2
GLYCINE

A separate pathway leading to serine from glyoxylate has been suggested in a variety of organisms. The postulated pathway is shown in Figure 2. The pathway from glyoxylate is present in E. coli, but the supply of serine from this source is inadequate to allow growth at the rate observed when glycine or serine is provided (30). Although the pathway from glyceric acid has been demonstrated in plants, the conversion of glycolate to serine appears to be the major pathway for serine biosynthesis in wheat leaves (31). Early studies supported the view that serine precedes glycine in the pathway utilized by baker's yeast (32), but more recent studies using short-term isotopic labeling indicate that the pathway from glycolate to serine predominates (33). In Neurospora, Wright reported that a serine-glycine auxotroph investigated by her grew better on glycine or glyoxylic acid than on serine (34,35). Combépine and Turian showed that glyoxylate-2-C^{14} was converted to glycine by crude extracts of Neurospora (36), but ruled out the pathway as a major source of serine by studies using serine-requiring mutants (37). These two studies on serine metabolism in Neurospora were thus contradictory, and additional investigation seemed warranted to determine whether Neurospora utilizes the pathway in which serine is formed from intermediates of glycolysis or whether serine is formed from glycine derived from glyoxylate.

The work described in this thesis sought to answer two questions:

Figure 2. Serine biosynthesis from glyoxylate. Glyoxylate may arise from intermediates in the tricarboxylic acid (TCA) cycle, via the reaction sequence known as the "glyoxylate shunt." Isocitric acid is shunted from the TCA cycle by isocitrate lyase, which splits isocitric acid to form succinic acid and glyoxylic acid. Alternatively, glyoxylate may arise from glycolic acid, which is formed from carbohydrate metabolism.


CITRIC ACID
HOCHCOOH
CHCOOH
CH2COOH
ISOCITRIC ACID
HOOC–CH2CH2COOH
SUCCINIC ACID
HOCH2COOH
OCH–COOH
GLYCOLIC ACID
GLYOXYLIC ACID
[NH3]
transamination
NH2CH2COOH
GLYCINE
"C1"
HOCH2CHCOOH
NH2
SERINE

(1) Why does P110 synthesize L-amino acid oxidase and NADase when growing on minimal medium, and what does this imply about the control of these enzymes in wild-type cultures?

(2) What is the nature of the biochemical lesion in P110 which results in its serine or glycine requirement? Does this lesion indicate the major pathway for serine biosynthesis in Neurospora?

MATERIALS AND METHODS

Strains

Neurospora crassa wild-type strain ST 74A was used extensively during these studies. The mutant P110, first described by John C. Urey (7), was derived by uv-irradiation of ST 74A. A sexual reisolate of P110 was used for some experiments. This strain, called II6a;*cot*, which carries the colonial temperature-sensitive (*cot*) marker, was derived from a cross between P110A and strain C102-8025-1a;*cot*, kindly provided by Mary B. Mitchell from the *Neurospora* stocks of the Caltech Division of Biology.

For genetic studies, the strains used were C102-R-7a;*cot*, and C102-15300-4-2A,*al-2*;*cot*, provided by Mary B. Mitchell, and the multiple mutant stock *leu-2*,*pan-1*,*mat* (37501,5531,B57); *a* (FGSC #335) from the Fungal Genetics Stock Center.

Heterocaryon studies utilized strain 37811a;*lys*, strain *arg-5a*, (FGSC #480), and a sexual reisolate of P110 carrying the *leu-2* and *pan-1* markers, called XXVI-0-24-6a; *leu-2*,*pan-1*. The lysineless strain was provided by Mary B. Mitchell and the arginineless strain was provided by D. Hugh Morgan.

Growth studies were carried out with strains obtained from the Fungal Genetics Stock Center: H605 *ser-1* (FGSC #116), C127 *ser-1* (FGSC #1126), and 47903 *ser-3* (FGSC #1213). The strain 65004 *ser-2* (FGSC #82) was also obtained, but this "mutant" grew better on minimal medium than on serine-supplemented medium.

Limited use was made of strains 38706A;me-1, C167 R_3 tyr, 35203a;ad-3B, C140 his-3, H98 me-2, 51504hs, and 16117 A;iv-1, all generously provided by Helen Macleod Feldman, and strains 46004A; arg-1 and arg-8A from D. Hugh Morgan.

Chemicals and Stains

Uniformly labeled C^{14} α-ketoglutarate (3.2 mc/mmole) and glycine-UL-C^{14} (5.0 mc/mmole) were obtained from Calbiochem. Nonlabeled compounds used were of reagent grade quality, except table sugar, which was used routinely as the sucrose in minimal medium.

In the assay of L-amino acid oxidase, sodium arsenate, dibasic, from J. T. Baker Chemical Company was found to contain an unidentified compound which interfered with the assay. Sodium arsenate from Baker and Adamson was therefore used routinely in these studies.

Dowex 1-X1, 200-400 mesh, used for anion exchange chromatography, was obtained from J. T. Baker Chemical Company. Before use, the reagent was washed with 4N HCl, 10 ml/gm resin. The acidified resin was rinsed with distilled water until the eluant pH was approximately 7. Next the resin was washed with 2N NaOH, 10 ml/gm, followed by a distilled water rinse. The resin was used in the OH^- form.

Dowex 50W-X4, 200-400 mesh, used for cation exchange chromatography, and Dowex 50W-X8, used to isolate glutamic acid from the phosphoserine transaminase reaction mixture, were also obtained from J. T. Baker Chemical Company. These resins were washed in a manner

identical to that used for Dowex 1, except that the NaOH wash was first and the HCl wash was second. These resins were used in the H^+ form.

Biogel P 10 was obtained from Calbiochem.

Sephadex G 200 was obtained from Pharmacia Fine Chemicals, Inc.

The scintillation fluid used during isotope studies was prepared from Spectrafluor-PPO-POPOP concentrated liquid scintillator from Amersham/Searle. Prepared as directed, the scintillation fluid contained 4 gm PPO and 50 mg POPOP per liter of toluene.

To locate amino acids on paper after high voltage electrophoresis or chromatography, a cadmium-ninhydrin stain was used. The stain was prepared by adding 24 ml of a stock solution of cadmium acetate (200 mg cadmium acetate in 20 per cent acidic acid) and 2 gm ninhydrin to 200 ml acetone. This volume of reagent was just enough to dip one 46 cm x 180 cm strip of Whatman #3MM paper used for high voltage electrophoresis.

Maintenance and Growth of Neurospora Cultures

Permanent stock cultures were maintained on anhydrous silica gel (38). Vegetative cultures were maintained on agar slants of Horowitz complete medium (39).

For most experiments, cultures were grown without shaking at 25°C in 125 ml Erlenmeyer flasks containing 20 ml of Vogel's minimal medium N salts (40) and 2 per cent sucrose. Each flask was inoculated

with 3 drops of conidial suspension. For determination of dry weight, the mycelial pads were fished from the medium with a spatula, blotted dry between sheets of filter paper, and dried overnight in a drying oven at 60-70°C.

For preparation of the enzymes involved in synthesis of serine from the products of glycolysis, cultures were grown in Westergaard-Mitchell medium (11) containing 1.5 per cent glucose or fructose. Fifty ml of this medium were used to wash all the conidia from a 5-7 day old culture grown on 20 ml solid complete medium in a 125 ml Erlenmeyer flask. The conidial suspension was filtered through glass wool before using it to inoculate 700 ml of minimal medium contained in a 2 liter low form culture flask. The wild-type was harvested after 19 hours on a reciprocal shaker at 25°C, while the mutant was grown shaking for 43 hours.

Derepression of Cultures

Production of L-Amino Acid Oxidase for Enzyme Purification Studies

To derepress tyrosinase and L-amino acid oxidase in wild-type, pads from stationary cultures were washed twice with 20 ml sterile distilled water and were then suspended on 5 ml sterile Vogel's salts containing 2.5 μg cycloheximide. Flasks were incubated for 48 hours at 25°C, without shaking and in the dark.

Derepression by the Pall Method

To study the effects of keto acids on derepression of tyrosinase and L-amino acid oxidase, wild-type cultures were derepressed by the method of Pall (41). Cultures were grown in 125 ml Erlenmeyer flasks containing 20 ml 1/2 X Vogel's salts and 1/2 per cent sucrose. Flasks were incubated for 48 hours at 25°C, without shaking and in the dark. To initiate derepression, 4 μg cycloheximide or 2 mg DL-ethionine was added to each flask. Incubation was continued for an additional 48 hours on a reciprocal shaker (80 strokes/minute) at 25°C.

Extraction of Soluble Enzymes

Mycelia were harvested at room temperature on a Buchner funnel with suction. Excess medium was removed by rinsing the mycelium briefly with distilled water. The mycelial pad was blotted dry between sheets of filter paper or paper towels. Pads for L-amino acid oxidase, tyrosinase, and NADase assay were routinely wrapped in aluminum foil and stored in a deep freezer until the pads were to be extracted. Such pads were weighed just before grinding with sea sand in a cold mortar and pestle. These enzymes were usually extracted with 10 volumes 0.1M sodium phosphate buffer, pH 6. Very small pads were extracted with up to 100 volumes of buffer. The mixture was spun in a Servall centrifuge at 12,350g for 15 minutes. The supernatant was assayed for enzyme activity.

Mycelia used for assay of the serine biosynthetic enzymes were extracted immediately after harvesting and weighing. Glyceric acid

dehydrogenase and phosphoglyceric acid dehydrogenase were extracted from the ground mycelium with 5-10 volumes 0.01M Tris-HCl, pH 7.5. The crude extract was centrifuged at 12,350g for 15 minutes. The low speed supernatant was centrifuged at 100,000g for 1 hour in a Spinco model L-2 centrifuge. The 100,000g supernatant was assayed for dehydrogenase activity. The second centrifugation was reported by Sojka and Garner (25) to reduce the high background level of NADH oxidation. My results confirmed their findings. For phosphoserine phosphatase assay, low speed supernatants were precipitated with solid $(NH_4)_2SO_4$ added to 70 per cent saturation at 4°C. The precipitate was pelleted by centrifugation, rinsed with cold buffer, and suspended in 0.01M Tris-HCl, pH 7.5. The solution was assayed for phosphatase activity. Phosphoserine transaminase and serine transaminase were extracted from the ground mycelium with 5 volumes 0.01M potassium phosphate buffer, pH 8. The mixture was spun at 12,350g for 15 minutes. The supernatant was applied to a small (2.5 cm x 13 cm) Biogel P 10 column which had been equilibrated with 0.01M potassium phosphate buffer. The protein peak eluted with the same buffer was assayed for transaminase activity.

Enzyme Assays

L-Amino Acid Oxidase

L-Amino acid oxidase activity was measured by the deamination of phenylalanine as described previously (8). The reaction mixture was identical to that described loc. cit., with the exception that the reaction was initiated by the addition of 0.25 ml of a 0.02M solution of

L-phenylalanine. The reaction was terminated by adding 1 ml 20 per cent (W/V) metaphosphoric acid. Precipitated proteins were removed by centrifugation. The amount of phenylpyruvate product in the supernatant was determined by the enol-borate method of Lin et al. (42). Phenylpyruvate-enol-borate (ϵ_{mmolar} = 9.15) was measured at 300 mμ in a Beckman DU-2 spectrophotometer. All data were converted to Enzyme Commission Units per gram wet weight of mycelium.

Tyrosinase

Tyrosinase was measured by the conversion of DOPA to dopachrome as described by Horowitz et al. (43). Formation of dopachrome was followed colorimetrically on a Klett-Summerson colorimeter with a No. 42 filter. The measured absorbance of dopachrome was converted to Enzyme Commission Units as described previously (44). All data are expressed as Enzyme Commission Units per gram wet weight of mycelium.

Nicotinamide Adenine Dinucleotide Glycohydrase

Nicotinamide adenine dinucleotide glycohydrase (NADase) activity was assayed using a NAD-cyanide reaction as described by Kaplan et al. (45). The reaction mixture was identical to that described loc. cit., with the exception that 0.1M sodium phosphate buffer, pH 7.2, replaced 0.1M KH_2PO_4. The NAD-CN complex (ϵ_{mmolar} = 6.3) formed was measured on a Beckman DU-2 spectrophotometer set at 325 mμ.

3-Phosphoglyceric Acid Dehydrogenase and Glyceric Acid Dehydrogenase

3-Phosphoglyceric acid dehydrogenase was measured by the disappearance of reduced nicotinamide adenine dinucleotide (NADH) according to the method of Umbarger et al. (29). Approximately 1 mg protein from a crude extract was assayed in a 3.3 ml reaction mixture. The protein concentration was determined by means of the biuret reaction (47). Glyceric acid dehydrogenase was assayed by substituting hydroxypyruvic acid for phosphohydroxypyruvic acid in the reaction mixture. Oxidation of NADH was followed spectrophotometrically in a Cary model 15 recording spectrophotometer at 340 mμ. A high level of NADH oxidase activity was observed in all extracts in the absence of added substrate. Data are expressed as μmoles NADH oxidized per mg protein per hour in the presence of added substrate minus the background activity expressed in the same units.

Phosphoserine Transaminase and Serine Transaminase

Phosphoserine transaminase and serine transaminase activities were assayed as described by Sojka and Garner (25). Enzyme activity is measured by the formation of radioactive glutamic acid by transamination of labeled α-ketoglutaric acid in the presence of enzyme and phosphoserine or serine. The reaction mixture contained 500 μmoles potassium phosphate buffer, pH 8; 20 μmoles L-serine (or 40 μmoles DL-O-phosphoserine); 20 μg pyridoxal phosphate; and 0.06 μmole C^{14} α-ketoglutarate having an activity of 4.2×10^5 cpm. This mixture was incubated at 26°C for several minutes before the reaction was begun by adding

approximately 5 mg protein from a crude extract prepared as described in this thesis. Total volume of the reaction mixture was 5 ml. The reaction was stopped by adding 1 ml samples of the reaction mixture to 0.1 ml of cold 50 per cent TCA. The precipitated protein was removed by centrifugation. A 0.75 ml aliquot of the supernatant was applied to a small Dowex 50W-X8, 200-400 mesh column in the H^+ form. These columns were made by packing a glass-wool plugged Pasteur disposable pipette with approximately 2 ml of Dowex. After application of the sample, the column was rinsed with 20 ml distilled water. Amino acids were eluted with 8 ml 6N NH_4OH. Samples were evaporated to dryness in a vacuum desiccator. The residue was dissolved in 0.2 ml distilled water. A 0.1 ml aliquot of this solution was applied to a small circle of Whatman #1 filter paper and evaporated to dryness. The paper was placed into a scintillation vial, 5 ml of scintillation fluid was added, and the samples were counted in a Beckman CPM 200 Scintillation Counter. Enzyme activity is expressed as μmoles glutamic acid formed per mg protein per hour.

Phosphoserine Phosphatase

Phosphoserine phosphatase was assayed by a slight modification of the method described by Ames (48). Enzyme activity is measured by the formation of inorganic phosphate from phosphoserine in the presence of enzyme. The reaction mixture contained 200 μmoles Tris-HCl, pH 7.5, 50 μmoles $MgCl_2$, and 20 μmoles DL-O-phosphoserine. The reaction mixture was incubated several minutes at 25°C before the reaction was begun by

adding approximately 1 mg protein from a crude extract. Total volume of the reaction mixture was 3 ml. The phosphatase reaction was stopped by adding 0.7 ml of the reaction mixture to 2.3 ml phosphatase mix (the mix contains 6 ml 10 per cent ascorbic acid and 36 ml 0.42 per cent ammonium molybdate·4 H_2O in 1N H_2SO_4). This solution was incubated for 20 minutes at 45°C. The absorbance of the reduced phosphomolybdate complex was read at 820 mμ in a Beckman DU-2 spectrophotometer. A solution containing 0.01 μmole inorganic phosphate gives an OD_{820} of 0.260 (48). Enzyme activity is expressed as μmoles phosphate formed per mg protein per hour.

Extraction of Intracellular Free Amino Acid Pools

Pads from cultures grown on Vogel's minimal medium (40) were harvested over a Buchner funnel, with suction. Excess medium was removed by rinsing the pads briefly with distilled water. Each pad was extracted in 10 ml boiling distilled water in a boiling water bath for 30 minutes. Debris was removed by centrifugation, and the supernatant was analyzed for individual amino acids as described below.

High Voltage Paper Electrophoresis and Chromatography of Amino Acids

Solutions containing the intracellular free amino acid pool extracted as described above were evaporated to dryness in a vacuum desiccator. The residue was dissolved in 0.5 ml distilled water. Ten to twenty μl aliquots of the sample were applied with a Beckman sample applicator (cat. no. 320005) to Whatman #3MM paper (46 cm x 180 cm),

and the amino acids were separated by electrophoresis. Electrophoresis was carried out in 6.7 per cent formic acid, pH 1.6, for 105 minutes at 7800 volts in a Gilson Model DW Electrophorator. The paper containing the separated amino acids was dried and the positions of the amino acids were determined by dipping the paper in cadmium-ninhydrin reagent. To estimate the concentrations of individual amino acids, the colored zones were cut from the large sheet and eluted. Each colored zone, or spot, was cut into strips to fit a 13 x 100 mm test tube. Color was eluted by adding 2 ml absolute methanol, stoppering the tube, and shaking for 2 hours at 25°C. The decanted solution was read against a methanol blank at 500 mμ. Each reading was converted to mμmoles amino acid present, using a set of empirically determined factors (49).

In certain experiments, the positions of labeled amino acids were determined by counting the unstained strip of separated amino acids on a Nuclear Chicago Actigraph II. The strips were then stained with cadmium-ninhydrin reagent, and the locations of radioactivity were correlated with the positions of the amino acid spots.

To separate the amino acids by chromatography, 5 μl samples of the amino acid solutions were applied to Whatman #3MM paper (46 cm x 53 cm). The chromatogram was developed for 12 hours with 77 per cent ethanol. After drying, the paper was dipped in cadmium-ninhydrin stain. In some cases, an unstained strip containing the separated amino acids was cut from the paper, sewn to a fresh sheet of Whatman #3MM paper and the amino acids were further separated by high voltage electrophoresis. In some experiments the sequence of steps was reversed.

This two-dimensional separation of the amino acids gave a clear picture of individual amino acid pool sizes.

Genetic Analysis

All crosses were performed on Westergaard-Mitchell medium (11) containing 2 per cent sucrose. Crosses I (P110A x C102-R-7a;cot) and II (P110A x C102-8025-1a;cot) and XXIV (II6a;cot x C102-15300-4-2A, al-2;cot), were prepared by coinoculation of agar plates with appropriate conidia. Cross XXVI (leu-2,pan-1,mat;a x P110A) was prepared by inoculating the triple mutant onto an agar slant of Westergaard-Mitchell medium supplemented with 0.2 mg/ml L-leucine and 10 µg/ml calcium pantothenate. After 7 days, the slant was fertilized with P110 conidia.

All crosses were incubated in the dark at 25°C.

Formation of Heterocaryons

One heterocaryon was prepared by coinoculating an agar slant of Westergaard-Mitchell medium with conidia of XXVI-0-24-6a;leu-2,pan-1 and arg-5a. A conidial suspension prepared from this presumed heterocaryon was plated onto Westergaard-Mitchell medium supplemented with 10 µg/ml calcium pantothenate. Hyphal tips were isolated onto slants of the same medium after the plates had been incubated at 25°C for 12-24 hours. Individual isolates were labeled H2-1, H2-2, etc.

A second heterocaryon, H3, was prepared by coinoculating a plate of Vogel's medium with equal volumes of conidial suspension of XXVI-0-24-6a;leu-2,pan-1 and 37811a;lys. A third heterocaryon, H4,

was prepared by coinoculating a plate of Vogel's medium with 5 drops of conidial suspension of 3781la;lys and 1 drop of conidial suspension of XXVI-0-24-6a;leu-2,pan-1. Control plates were inoculated with a single conidial suspension. All plates were left at room temperature for 6 hours, to allow adsorption of the suspending liquid into the agar plate. The plates were incubated at 25°C for 12 hours. Because no growth on any plate was observed after 12 hours, plates were transferred to 30°C to hasten growth. Hyphal tips were isolated from H4 at 30 hours and 36 hours after the shift to 30°C. Hyphal tips from H3 were isolated 36 hours and 48 hours after the shift to 30°C. Hyphal tips were isolated onto slants of Vogel's minimal medium and were incubated at 30°C. No growth occurred on either control plate within 72 hours after the shift to 30°C.

RESULTS

Genetic Analysis of the Mutant P110

Inheritance of Constitutive Synthesis of L-Amino Acid Oxidase

To determine whether constitutive formation of L-amino acid oxidase was inherited, mutant P110 was first crossed to two strains, C102-R-7a;*cot* (cross I), and C102-8025-1a;*cot* (cross II). These strains were used because each strain carries the colonial temperature-sensitive marker, *cot*. A *cot*-carrying strain with the properties of P110 was desired for the isolation of mutants defective in L-amino acid oxidase production (see Appendix). Random spores from the two crosses were spread on plates of complete medium. Following heat shock at 57-58°C for one hour, the spores were incubated at 25°C. Germinated ascospores were isolated onto slants of complete medium 13-16 hours after the beginning of incubation. Isolates were incubated at 35°C to test for the *cot* characteristic. The results indicated selection against the *cot*$^+$ progeny:

cross I: 26 *cot*: 13 *cot*$^+$

cross II: 31 *cot*: 14 *cot*$^+$

Each isolate was analyzed for production of L-amino acid oxidase when grown on Vogel's minimal medium. The locus determining the constitutive synthesis of oxidase in these strains is designated *P110*. The results shown in Table I indicate that very few *cot* progeny were obtained which produced oxidase when grown on minimal medium. This

TABLE I

Production of L-amino acid oxidase by progeny from P110A x C102-R-7a;cot (cross I) and P110A x C102-8025-1a;cot (cross II)

	Number of Progeny
Cross I	
Parental types:	
cot; +	25
+ ;P110	9
Recombinant types:	
cot;P110	1
+ ; +	4
Cross II	
Parental types:	
cot; +	30
+ ;P110	12
Recombinant types:	
cot;P110	1
+ ; +	2

result and the excess of parental types compared to recombinant types suggested that P110 is on the same linkage group as cot (IV), and that P110 progeny are selected against. Consistent with the proposed negative selection was the observation that P110 and all strains which produced oxidase when growing on minimal medium were characterized by slow growth. The relationship between slow growth and high L-oxidase activity was first observed by Burton (3).

Inheritance of Constitutive Synthesis of NADase

The mutant P110 differs from ST 74A from which it was derived in the constitutive synthesis of NADase, as well as L-oxidase, when growing on minimal medium (7). That the production of both enzymes resulted from the same mutation had to be considered in view of the fact that both characters arose simultaneously in P110 following uv-irradiation of ST 74A. At the time the present studies were carried out, John Urey of our group was interested in the control of NADase synthesis and investigated the inheritance of NADase constitutivity among the P110 progeny. Scoring of the NADase constitutive character is sometimes confused by the presence of conidia on pads grown on minimal medium. All conidia, including those of wild-type strains, contain such a high level of NADase that even very limited conidiation can result in scoring a non-constitutive as an NADase constitutive. Therefore, tests of the progeny were performed on cultures grown on Vogel's minimal medium containing 0.8 per cent Tween 80 (polyoxyethylene sorbitan monooleate Mefford), a compound which prevents conidiation

by such cultures (50). Both the mycelial pads and media were assayed for NADase. A comparison of the progeny determined by Urey to be high producers of NADase under these conditions and progeny which produced L-amino acid oxidase when grown on minimal medium indicated that the two properties were genetically separable. Because these results were unexpected, I reinvestigated the inheritance of NADase production. The parental strain C102-R-7a;cot produced a moderate amount of NADase when grown on minimal medium and Tween 80, so cross I was not analyzed for inheritance of the character. Among the cross II progeny which produced L-amino acid oxidase when growing on minimal medium, two of the twelve strains analyzed produced little if any NADase when growing on minimal medium and Tween 80.

As indicated, the media on which the cultures were grown for determination of production of the two enzymes were different. Attempts to compare synthesis of the two enzymes in cultures grown on minimal medium and Tween 80 were precluded because no culture produced L-oxidase under these conditions (7). The absence of oxidase activity in these extracts is not due to the presence of an inhibitor. Adding extract from a culture grown on Tween 80 did not reduce the activity measured in an extract from P110 grown on minimal medium.

The fact that Tween 80 prevents conidiation suggested that the oxidase might be a conidial enzyme. The presence of L-oxidase in pads of P110 grown on minimal medium could then be attributed to an earlier production of conidia by the mutant than by wild-type growing on minimal medium. To test the suggestion that the oxidase gene was active in

conidia, a dense conidial suspension of P110 was disrupted with glass beads in a disintegrator (McDonald Instrument Company) for one minute. Conidia of ST 74A were treated similarly. Debris was removed by centrifugation. The supernatant was assayed for L-amino acid oxidase, NADase, and protein. Both protein and NADase were present in the extract, but no oxidase activity was detected in P110 or wild-type in an assay mixture containing 1 mg protein from the conidial extract. An equivalent amount of protein from a crude mycelial extract of P110 would have contained a high level of oxidase.

Despite the problems involved in studying the NADase in cultures grown on Vogel's medium, a random group of progeny was studied in this way to compare the production of NADase and oxidase under comparable conditions. The results again indicated that some progeny produced the oxidase without producing NADase and vice versa. These results indicated either that production of the two enzymes is genetically separable or that more than one gene affects the constitutive synthesis of NADase or oxidase.

Evidence for the Single-Gene Inheritance of Constitutive Synthesis of L-Oxidase

The random spore data did not demonstrate whether the synthesis of L-amino acid oxidase on minimal medium was inherited as a single gene. Two additional crosses showed that the characteristic was simply inherited. Cross II was repeated and individual asci were dissected. Isolated spores were heat-shocked at 55°C for 45 minutes. All isolates

were incubated at 25°C. Spore germination was poor, so that only five asci were obtained in which all four spore pairs were represented. Nevertheless, these asci were consistent with single-gene inheritance of the character (Table II). Despite variability between duplicate pads, strains which produce a high level of oxidase when growing on minimal medium (P110) are easily distinguishable from wild-type strains.

The purpose of the next cross was to determine whether P110 is on linkage group I, on which the tyrosinase structural gene had been mapped (51). Several observations had suggested that tyrosinase and L-amino acid oxidase might be controlled by genes associated in an operon (8). If the synthesis of the oxidase resulted from an alteration of the oxidase structural gene, or its associated (hypothetical) operator region, and if the oxidase gene and the tyrosinase gene were linked, then P110 would be expected to map on linkage group I. For this cross, a sexual reisolate of P110, called II6a;cot, was crossed to C102-15300-4-2A,al-2;cot. The reisolate was used because it did not synthesize NADase when the culture was growing on Vogel's medium and Tween 80. If the latter characteristic had been responsible for the poor viability of P110 progeny in the previous crosses, then this cross would be expected to show good viability. In this cross, random spores were spread on agar plates of Vogel's medium. Spores were heat-shocked and incubated at 32°C overnight. Germinated spores were isolated onto complete medium and incubated at 25°C. Each culture was tested in duplicate for synthesis of the oxidase when growing on Vogel's medium. This characteristic segregated independently from the

TABLE II

Types of asci obtained from cross P110A x C102-8025-1a;cot

Spore no.	cot/+	P110/+	L-oxidase, ECU/gm	
			Set I	Set II
Ascus I, ordered, 7 spores germinated				
1	+	+	0.78	0.75
2	+	+	0.48	0.42
3	cot	+	0.58	0.25
4	cot	+	0.40	0.36
5	+	P110	14.2	8.7
6	+	P110	13.6	11.6
8	cot	P110	25.4	16.7
Ascus II, ordered, 7 spores germinated				
2	cot	P110	11.3	19.5
3	+	P110	6.8	15.1
4	+	P110	6.8	10.6
5	cot	+	0.32	0.62
6	cot	+	0.44	0.56
7	+	+	0.51	0.78
8	+	+	0.51	0.59
Ascus III, ordered, 5 spores germinated				
2	+	P110	5.3	8.1
3	+	P110	3.9	6.0
4	+	P110	4.0	6.6
6	cot	+	0.68	1.0
7	cot	+	0.59	0.76
Ascus IV, unordered, 7 spores germinated				
	+	P110	6.6	5.9
	+	P110	4.3	3.3
	cot	+	0.19	0.19
	+	P110	4.6	4.0
	cot	+	0.25	0.14
	cot	+	0.21	0
	cot	+	0.27	0.07

TABLE II (continued)

Spore no.	cot/+	P110/+	L-oxidase, ECU/gm	
			Set I	Set II
Ascus V, unordered, 8 spores germinated				
	+	+	0.07	0.17
	cot	+	0.13	0.34
	+	+	0.04	0.12
	+	P110	2.6	3.6
	+	P110	3.8	4.4
	cot	P110	8.3	8.2
	cot	P110	9.4	12.8
	cot	+	0.15	0.64

albino conidial characteristic (al-2), as shown in Table III. Quite clearly, P110 was selected against. There are, however, as many albino progeny as wild-type conidiators which synthesize the oxidase. The data are consistent with the hypothesis that synthesis of the oxidase by strains growing on minimal medium (P110) is inherited as a single gene which is unlinked to markers on linkage group I. However, the data do not exclude the possibility that more than one gene is involved in the synthesis of L-amino acid oxidase by growing strains.

Mapping of P110 on Linkage Group IV Near the Centromere

As stated previously, preliminary results indicated that P110 might be on linkage group IV. Rather than map the locus by analyzing additional asci from cross II, a four-point cross was constructed between P110 A and the triple mutant leu-2, pan-1, mat;a, whose markers cover most of the right arm of linkage group IV. Twenty-eight asci were dissected, in 15 of which all 4 spore pairs germinated. Three spore pairs germinated in an additional 7 asci. Because the genotype of the missing spore pair can be inferred from the 3 known spore pairs, these asci were considered to be fully analyzed. Among the remaining asci, 2 spore pairs germinated in 3 cases, and 1 spore pair germinated in 3 cases. Quite often, asci were observed with 4:4 segregation of light and dark spores. Where noted, the light spores were later found to carry P110. A second heat shock given to the ungerminated spores about one week after the initial heat shock resulted in the germination of additional spores, many of which were P110,

TABLE III

Results of cross II6a;cot x C102-15300-4-2A,al-2;cot

	Number of progeny
Parental types:	
al-2; +	13
+ ; P110	5
Recombinant types:	
al-2; P110	7
+ ; +	17

suggesting that P110 spores mature more slowly than wild-type spores.

All progeny were tested at least twice for synthesis of L-amino acid oxidase when growing on minimal medium (or on leucine and/or pantothenate in the case of strains carrying leu-2 and/or pan-1). As in the previous crosses, progeny which produced the oxidase were characterized by slow growth on minimal medium.

A summary of the results obtained with the 22 fully analyzed tetrads is given in Tables IV and V. Because it is not known on which arm of the chromosome P110 is located, two analyses are shown. In one case, P110 was assumed to lie on the right side of the centromere. In the second case, P110 was placed on the left side of the centromere.

A map constructed by considering both centromere-gene distances and intergene distances is given below Table V. The locations of leu-2, pan-1, and mat are in reasonable agreement with published values (52, 53). Unfortunately, the data do not indicate whether P110 maps to the right or to the left of the centromere. A more precise location of P110 on linkage group IV could be obtained by crossing the mutant to the multiply marked strain col-4, pyr-1, pyr-3 (70007, H263, 37815t), (FGSC #390).

Heterocaryon Studies

Theoretically, several different types of mutations could lead to synthesis of L-amino acid oxidase by strains growing on minimal medium. Using the operon hypothesis as a model, P110 could be an

TABLE IV

Ordered tetrads from cross P110A x leu-2, pan-1, mat;a. The region within which an exchange has occurred is indicated by a Roman numeral. The particular strands involved in the exchange are denoted by the letters a, b, c, or d. The strands involved in the proximal exchange are arbitrarily designated bc.

Strand a	cent	P110	+	+	+	P110	cent	+	+	+
Strand b	cent	P110	+	+	+	P110	cent	+	+	+
Strand c	cent	+	leu-2	pan-1	mat	+	cent	leu-2	pan-1	mat
Strand d	cent	+	leu-2	pan-1	mat	+	cent	leu-2	pan-1	mat
Region		I	II	III	IV		I	II	III	IV

Tetrad structure	Number of tetrads observed		
P110 / P110 / + / + ; ± / leu-2 / leu-2 ; ± / pan-1 / pan-1 ; ± / mat / mat	9	No exchange	No exchange
P110 / P110 / ± / ± ; ± / leu-2 / + / leu-2 ; ± / pan-1 / ± / pan-1 ; ± / mat / ± / mat	2	Single II	Single II

TABLE IV (continued)

Tetrad structure				Number of tetrads observed		
P110 / P110 / + / +	+ / + / leu-2 / leu-2	+ / pan-1 / + / pan-1	+ / mat / + / mat	1	Single III	Single III
P110 / P110 / + / +	+ / + / leu-2 / leu-2	+ / + / pan-1 / pan-1	+ / mat / + / mat	1	Single IV	Single IV
P110 / + / P110 / +	+ / leu-2 / + / leu-2	+ / pan-1 / + / pan-1	+ / mat / mat / +	1	3-Strand Double Ibc, IVbd	3-Strand Triple Ibc, IIbc, IVbd
P110 / P110 / + / +	+ / leu-2 / + / leu-2	+ / pan-1 / + / pan-1	+ / mat / mat / +	2	3-Strand Double IIbc, IVbd	3-Strand Double IIbc, IVbd
P110 / + / P110 / +	+ / leu-2 / leu-2 / +	+ / pan-1 / pan-1 / +	+ / + / mat / mat	1	3-Strand Triple Ibc, IIbd, IVbc	3-Strand Triple Ibc, IIbd, IVbd

TABLE V

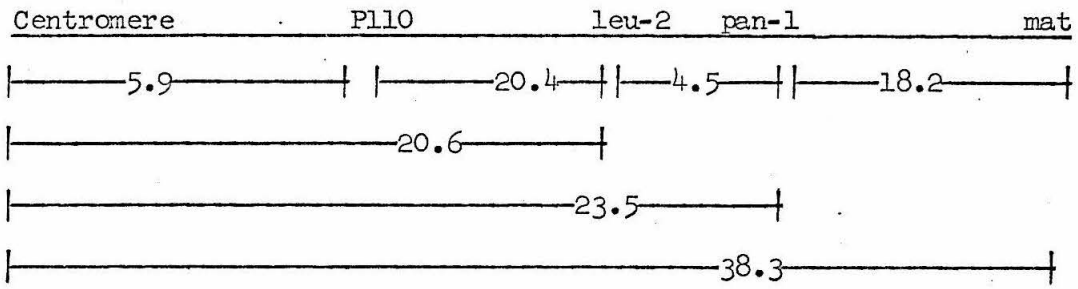
Unordered tetrads from cross P110A x leu-2, pan-1, mat;a.

Strand a	P110	+	+	+	
Strand b	P110	+	+	+	
Strand c	+	leu-2	pan-1	mat	
Strand d	+	leu-2	pan-1	mat	
Region		I	II	III	

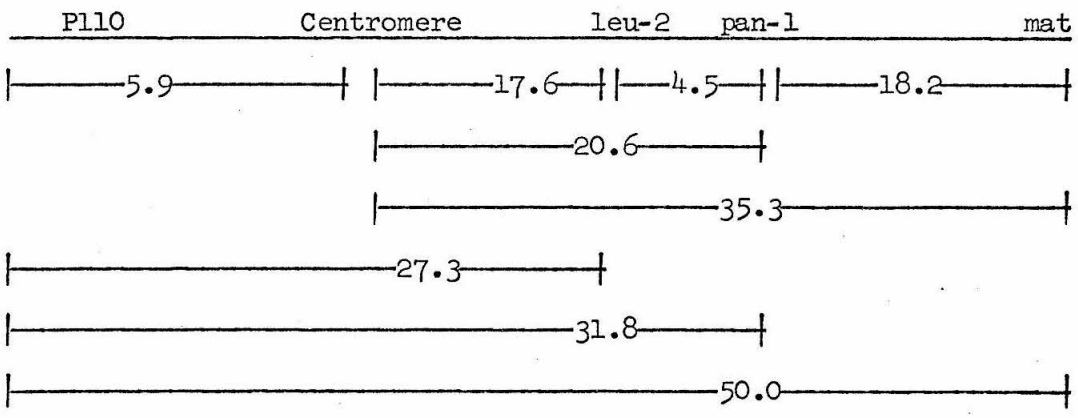
Tetrad structure				Number of tetrads observed	
P110	leu-2	pan-1	mat		
P110	+	+	+	1	Single I
+	leu-2	pan-1	mat		
+	+	+	+		
P110	+	pan-1	mat		
P110	+	+	+	1	Single II
+	leu-2	pan-1	mat		
+	leu-2	+	+		
P110	leu-2	pan-1	mat		
P110	+	+	+	2	Double Ibc, IIIbd
+	leu-2	pan-1	+		
+	+	+	mat		
+	leu-2	pan-1	mat		
P110	+	+	mat	1	Double Ibc, IIIac
P110	leu-2	pan-1	+		
+	+	+	+		

TABLE V-a

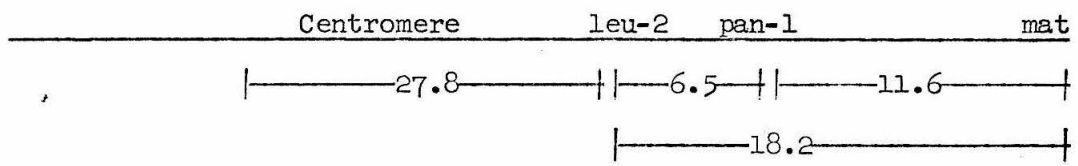
Maps calculated from data in Tables IV, V



or



Published map (52, 53)



operator-constitutive mutant, whose operator region no longer binds the repressor which prevents synthesis of the oxidase in wild-type. If tyrosinase and oxidase are controlled by the same repressor, P110 could be a mutant with an altered co-repressor, necessary for repression of the L-oxidase gene, but not for repression of the tyrosinase gene. If the two enzymes are controlled by two separate repressors, then P110 might result from a mutation in the oxidase-specific repressor. To reduce the number of these various possibilities, heterocaryons were prepared to learn whether the properties of P110 were dominant or recessive in the presence of wild-type nuclei (54). The results of these studies were unusual and remain unexplained.

Formation of a Heterocaryon Between an L-Oxidase Constitutive Strain and Arg-5a

The first heterocaryon prepared was H2 (XXVI-0-24-6a;leu-2, pan-1) (arg-5a). Individual hyphal tips were isolated as described in the Materials and Methods section. Two isolates were investigated with respect to synthesis of the oxidase on minimal medium and frequency of the two nuclear types present in the heterocaryon. Conidial suspensions were prepared and filtered through glass wool to remove mycelial fragments. The suspensions were diluted serially and plated on complete medium in which the glycerol had been replaced by 1 per cent L-sorbose and 0.1 per cent glucose. Sorbose was added to cause the conidial isolates to grow colonially. Complete medium was used to allow growth of all types of conidia; i.e. both homocaryotic and

heterocaryotic conidia. The same conidial suspension was used to inoculate flasks of minimal medium supplemented with pantothenate. Colonies which grew up on complete medium were tested for growth on three media: Westergaard-Mitchell medium was supplemented with (1) pantothenate, (2) leucine and pantothenate, or (3) arginine. Fifteen colonies were isolated from H2-4 and 22 colonies were isolated from H2-10. Two types of cultures were recovered: those which grew only on medium containing leucine and pantothenate, typical of XXVI-0-24-6, and cultures which grew on all three media. The latter cultures were assumed to represent heterocaryotic nuclei. No arg-5 colonies were recovered. The presumed heterocaryon and isolates which grew on liquid minimal medium plus pantothenate produced no L-oxidase. Conidial isolates which required leucine and pantothenate did synthesize the oxidase when grown on supplemented minimal medium. Thus, the properties of P110 appeared to be recessive in this heterocaryon.

Attempts to Recover Arg-5a Nuclei from Conidia Produced by the Heterocaryon

It was of interest to determine the nuclear ratio of XXVI-0-24-6 to arg-5 in the heterocaryon, in order to estimate what proportion of arg-5 nuclei was required to prevent synthesis of L-oxidase by the heterocaryon. A filtered conidial suspension of H2-10 was plated on complete medium and 118 colonies were isolated onto the three test media. Of these isolates, 113 grew only on leucine and pantothenate; 1 grew on all three media, and 4 isolates did not grow. No arg-5

colonies were recovered. To determine whether plating on complete medium might have suppressed growth of the arg-5 conidia, serial dilutions of filtered conidial suspensions of H2-4 and H2-10 were plated on Vogel's medium supplemented with 0.5 mg/ml L-arginine, 1 per cent L-sorbose and 0.1 per cent glucose. The suspension was also plated on similar plates which contained leucine and pantothenate in addition to arginine. From plates supplemented only with arginine, 40 colonies were isolated from H2-4 and 67 colonies were isolated from H2-10. Nineteen colonies from H2-4 and 15 colonies from H2-10 were isolated from plates containing all three supplements. All colonies isolated from arginine-supplemented plates grew on all three test media, indicating that such colonies were derived from heterocaryotic nuclei or from wild-type nuclei. Of the H2-4 colonies isolated from triply-supplemented plates, 11 cultures grew on all three test media, while 8 cultures grew only on leucine and pantothenate. Of the H2-10 colonies isolated from triply-supplemented plates, 14 cultures grew on leucine and pantothenate only; one culture grew on all media. No arg-5 nuclei were recovered.

The results obtained above could be explained by an extremely low frequency of arg-5 nuclei in the heterocaryon. To recover these arg-5 nuclei if they were present, filtration enrichment (55,56,57) was performed. Conidial suspension of H2-10 was grown on 500 ml Vogel's medium supplemented with leucine and pantothenate in a 1 liter Erlenmeyer flask on a reciprocal shaker at 25°C. As conidia germinated and grew, mycelium was filtered off by pouring the suspension through 4

layers of sterile gauze. After 6 days and 8 filtrations, aliquots of the filtrate were plated onto Vogel's medium supplemented with arginine, 1 per cent L-sorbose and 0.01 per cent glucose. Forty-five colonies were isolated from these plates and retested for growth on various supplements. All colonies tested grew only on medium containing leucine and pantothenate. Apparently, the colonies growing on arginine-supplemented plates were able to utilize the leucine and pantothenate carried over in the plated filtrate. Arg-5 cultures were recovered from a control flask containing conidial suspension from the heterocaryon and a small number of added arg-5 conidia, indicating that arg-5 conidia should have been recovered if they were present in the flask containing conidial suspension from the heterocaryon. Twenty-five of thirty colonies isolated from the control flask were arg-5 colonies.

Evidence Against Wild-type Nuclei in the Heterocaryon

The inability to recover arg-5 nuclei from the heterocaryon led to the suggestion that H2 might be composed of XXVI-0-24-6 and wild-type nuclei. The wild-type nuclei might have come from a chance reversion of the original arg-5 strain, or they might have come from contamination of the culture by wild-type laboratory stocks. To test this possibility, asci were dissected from the cross H2-4 x ST 74A. If H2-4 contained wild-type nuclei, some asci containing all wild-type spores should have been recovered. Nineteen ordered asci were isolated and analyzed. All asci showed segregation of the leu-2 and pan-1 markers. In order to

observe larger numbers of H2 progeny more readily, H2-4 and H2-10 were crossed to leu-2A. Random spores were spread onto plates of Vogel's minimal medium and medium supplemented with leucine and pantothenate. After heat shock, the plates were incubated at 25°C. No wild-type spores were observed on minimal medium, although 5 densely-populated plates of each cross were carefully scanned. In all, several hundred germinated spores were examined; all showed the truncated growth typical of mutant spores germinated without supplement. On the supplemented plates, all progeny spores grew quite well. No evidence was obtained for the nature of the second component of the H2 heterocaryon.

A Cross Between the Heterocaryon and Wild-type

The next possibility investigated was that arg-5 nuclei were excluded from conidia for unknown reasons, despite their presence in the heterocaryon. To test this idea, slants of H2-4 and H2-10 grown on Westergaard-Mitchell medium were fertilized with conidia of ST 74A, and arg-5 progeny spores were sought. Random spores were spread on plates of Vogel's medium or Vogel's medium supplemented with leucine and pantothenate. Heat-shocked spores were incubated at 25°C. Spores isolated from supplemented plates showed the truncated growth typical of mutant spores germinated without their supplement. This selection was designed to eliminate wild-type progeny and leu-2, pan-1 progeny. Ninety-one spores from H2-10 x ST 74A and 50 spores from H2-4 x ST 74A were isolated onto minimal medium supplemented with arginine. Of the 91 spores isolated from H2-10 progeny, 42 grew on minimal medium

supplemented with arginine. All 42 grew on minimal medium upon transfer, indicating that none of the cultures was arg-5. Similarly, 4 of the 50 spores isolated from H2-4 progeny grew on arginine, and all of these grew on transfer to minimal medium. As a control, 30 spores were isolated from unsupplemented plates of H2-10 x ST 74A onto medium containing leucine and pantothenate. All isolates grew on these supplements; 20 of the 30 isolates also grew well on minimal medium.

In summary, the first heterocaryon prepared grew on minimal medium and pantothenate and produced no L-amino acid oxidase. Two types of conidia were recovered from the heterocaryon: one required leucine and pantothenate and produced the oxidase when grown on supplemented medium; the second type grew on unsupplemented medium and produced no oxidase. The most reasonable explanation for these results is that arg-5a nuclei are present in the heterocaryon, but do not get into conidia or participate in crosses involving the heterocaryon. Not excluded, however, is the possibility that the heterocaryon is composed of a high fraction of XXVI-0-24-6 nuclei and a few nuclei of an unidentified component which prevents the synthesis of oxidase and compensates for the leucine requirement of XXVI-0-24-6.

Formation of a Heterocaryon Between an L-Oxidase Constitutive Strain and a Lysine-Requiring Strain

The unexpected results obtained with heterocaryon H2 led to the preparation of two heterocaryons between XXVI-0-24-6a;leu-2,pan-1, which synthesizes oxidase, and 37811a,lys, which does not synthesize oxidase

when grown on its supplement. One of these heterocaryons, H3, was prepared with equal volumes of conidial suspension from the two cultures. The second heterocaryon, H4, was prepared with 5 volumes of conidial suspension of 37811 and 1 volume of conidial suspension of XXVI-0-24-6. Hyphal tips were isolated as described under Materials and Methods.

One hyphal tip isolate from each heterocaryon was selected for determination of nuclear ratios. Filtered conidial suspensions of H3-1 and H4-1 were plated onto Vogel's medium supplemented with 0.2 mg/ml L-leucine, 10 μg/ml calcium pantothenate, 0.5 mg/ml L-lysine, 1 per cent sorbose and 0.01 per cent glucose. Plates were incubated at 30°C. Three and one-half days after inoculation, colonies were isolated onto three test media: (1) Vogel's minimal medium, (2) Vogel's medium supplemented with lysine, and (3) Vogel's medium supplemented with leucine and pantothenate. Forty colonies were isolated from H3-1 plates and 40 colonies were isolated from H4-1 plates. All isolates were incubated at 25°C. The results are summarized below:

Heterocaryon	Growth on indicated medium		
	Minimal	leu + pan	lys
H3-1	0	32	8
H4-1	0	28	12

Thus, both components of the heterocaryon were recovered, but no cultures which arose from heterocaryotic nuclei were recovered. The frequency of nuclei containing the leu-2,pan-1 markers was higher than

the frequency of lys nuclei, even in heterocaryon H4, which was prepared with an excess of lys conidia.

At the same time that conidial suspensions of the hyphal tips were plated to determine the nuclear frequencies, flasks of Vogel's minimal medium were inoculated with the same conidial suspensions. In addition, six other hyphal tip isolates of H3 and 4 isolates of H4 were similarly tested for synthesis of L-amino acid oxidase when grown on minimal medium. Cultures were grown for 5 days at 25°C. Wet weights varied from 17.5 to 80 mg, with an average of 27.7 mg. All isolates produced a high level of oxidase. The amount of oxidase produced varied from 2.8 ECU/gm wet weight to 15.7 ECU/gm.

The results with H3 and H4 were contrary to the results obtained with H2. In heterocaryons H3 and H4, synthesis of L-amino acid oxidase on minimal medium appeared to be a dominant characteristic, whereas heterocaryon H2 indicated that this characteristic was recessive.

The absence of heterocaryotic cultures among the conidial isolates from H3 and H4 suggested a possible explanation for these contradictory results. If XXVI-O-24-6 and 37811 lys formed an unstable heterocaryon in which XXVI-O-24-6 soon predominated, then liquid-grown cultures might represent cultures of XXVI-O-24-6 growing symbiotically with 37811 lys. The compounds needed by XXVI-O-24-6 for growth might be supplied by 37811 lys without preventing constitutive synthesis of oxidase. To obtain cultures in which the ratio of the two nuclei should be close to unity, heterocaryotic conidial isolates were sought. To look for heterocaryotic conidia from H3 and H4, filtered conidial

suspensions were plated onto sorbose minimal medium and medium supplemented with lysine, leucine, and pantothenate. Each of the two hyphal tip isolates, H3-1 and H4-2 showed about 100 colonies on the supplemented plates for each colony on unsupplemented plates. Hence, the frequency of heterocaryotic conidia was low, but heterocaryotic conidia were present. Colonies were isolated from unsupplemented plates onto slants of minimal medium. Seventy-one colonies were isolated from a supplemented plate inoculated with H3-1 onto the three test media. Of these colonies, 70 were leu-2, pan-1 and one colony was lys. From supplemented plates inoculated with H4-2, 123 colonies were isolated onto slants of the three test media. Of these colonies, 119 were leu-2, pan-1, and 4 did not grow.

Colonies isolated from H4-2 plated onto unsupplemented medium were tested for synthesis of L-amino acid oxidase on liquid minimal medium. These heterocaryons did not produce L-amino acid oxidase when grown on minimal medium, whereas the hyphal tip isolate which gave rise to these heterocaryotic conidial isolates did produce the enzyme. To test the possibility that the colonies which had grown on minimal medium were wild-type, conidial suspension of each isolate was plated onto minimal medium and minimal medium supplemented with leucine, pantothenate, and lysine. Each isolate contained 10-100 conidia which grew on supplemented plates for each conidium which grew on minimal plates. Colonies isolated from one culture plated on supplemented media were tested for their requirements. Eighty-one were leu-2, pan-1, while 5 were heterocaryotic. Thus, the cultures which grew on minimal medium

were not wild-type contaminants, but were heterocaryons containing leu-2, pan-1 and/or lys nuclei.

Contrary to the results with H4-2, all of the 6 conidial isolates from H3-1 plated on minimal medium produced high levels of L-amino acid oxidase. Conidial suspension from one of these cultures was tested for the frequency of leu-2, pan-1 and lys nuclei and for synthesis of L-amino acid oxidase. The culture had a ratio of 30 leu-2, pan-1 nuclei: 1 lys nucleus: 1 heterocaryotic nucleus. Cultures grown from this conidial suspension did not synthesize the oxidase when grown on minimal medium.

In summary, variable results with respect to synthesis of L-amino acid oxidase were obtained with heterocaryons prepared with 37811a; lys and XXVI-0-24-6a; leu-2, pan-1, which synthesizes oxidase. Hyphal tip isolates of H3 and H4 produced the enzyme. Heterocaryotic conidial isolates of H4 did not produce the enzyme, while similar isolates of H3 did produce the enzyme. After vegetative transfer of one heterocaryotic conidial isolate of H3, it no longer synthesized the oxidase. The synthesis of L-amino acid oxidase by the culture is not directly related to the frequency of nuclei contributed by the constitutive parent as determined by plating conidia. A very high frequency of leu-2, pan-1 nuclei was found in heterocaryons not synthesizing the oxidase. In all three heterocaryons prepared, the leu-2, pan-1 nucleus was found to predominate, even in cultures arising from a single conidium. The reason for this predominance of one nuclear type is unknown. Pittenger and Brawner (58) observed a similar imbalance in nuclear ratios in

certain heterocaryons and found that the imbalance was controlled by a nuclear gene which they called I. In heterocaryons with a proportion of I nuclei ≥ 0.3, multiplication of i nuclei was inhibited, leading to a nonadaptive increase in I nuclei. Conidia isolated from such heterocaryons were predominantly I, although a few heterocaryotic conidia were recovered.

Ryan and Lederberg (59) observed that leucineless nuclei predominate in a heterocaryon between leucineless and wild-type strains if the heterocaryon is grown on leucine supplemented medium, while wild-type nuclei predominate in the same heterocaryon grown on minimal medium. This observation cannot explain the predominance of leu-2, pan-1 nuclei observed in the heterocaryons studied here, which were maintained on minimal medium.

Perhaps the results obtained with the heterocaryons can be attributed to the fact that nuclear ratios were determined by plating conidia from a culture which was used as the inoculum for the cultures later analyzed for the presence of L-amino acid oxidase. The nuclear ratio present in the culture grown on liquid may have been significantly different from the initial conidial ratio. No attempt was made to determine the nuclear ratio present in cultures which had been grown up on liquid medium.

Derepression of Tyrosinase in the L-Oxidase Constitutive Strain

The majority of conditions which derepress L-amino acid oxidase in wild-type cultures also derepress tyrosinase. The mutant P110

grown on minimal medium synthesizes L-amino acid oxidase, but not tyrosinase. That the tyrosinase gene was intact in the mutant was demonstrated by the derepression of tyrosinase under conditions which derepress the wild-type (7). A sexual reisolate of P110, called II6a;cot, was used for the experiment shown in Figure 3. The oxidase activity did not change significantly during derepression of the tyrosinase. The finding that only one of the two enzymes in the postulated operon (8) was affected argued against a mutation in a common regulator gene.

A Comparison of Partially Purified L-Amino Acid Oxidase from Derepressed Wild-type and from P110 Grown on Minimal Medium

If tyrosinase and oxidase were not controlled by two genes associated in an operon, but by two genes controlled by a common repressor, then a mutation affecting only one of the enzymes could result from alteration of one of the two (hypothetical) operator regions. If this were the case, then the enzyme synthesized might show a change in its physical properties.

When the thermostability of the oxidase produced by P110 grown on minimal medium was compared with the oxidase synthesized by derepressed ST 74A, a striking difference was observed (Figure 4). Because the thermostability of an enzyme can be affected by accompanying impurities, the two preparations were partially purified. In preliminary studies, the oxidase was purified by the method described by Fling

Figure 3. Derepression of tyrosinase in the L-amino acid oxidase constitutive strain, II6a;cot. Cultures were grown in 125 ml Erlenmeyer flasks containing 20 ml 1/2X Vogel's medium (40) and 1/2 per cent sucrose. The cultures were incubated without shaking at 25°C for 7 days. Derepression was initiated by the addition of 2 mg DL-ethionine per flask. The cultures were incubated at 25°C on a reciprocal shaker (80 strokes/minute) during derepression. Individual flasks were harvested at different times after the addition of ethionine and analyzed for tyrosinase and L-amino acid oxidase. Enzyme activities are given as Enzyme Commission units per gram wet weight of mycelium.

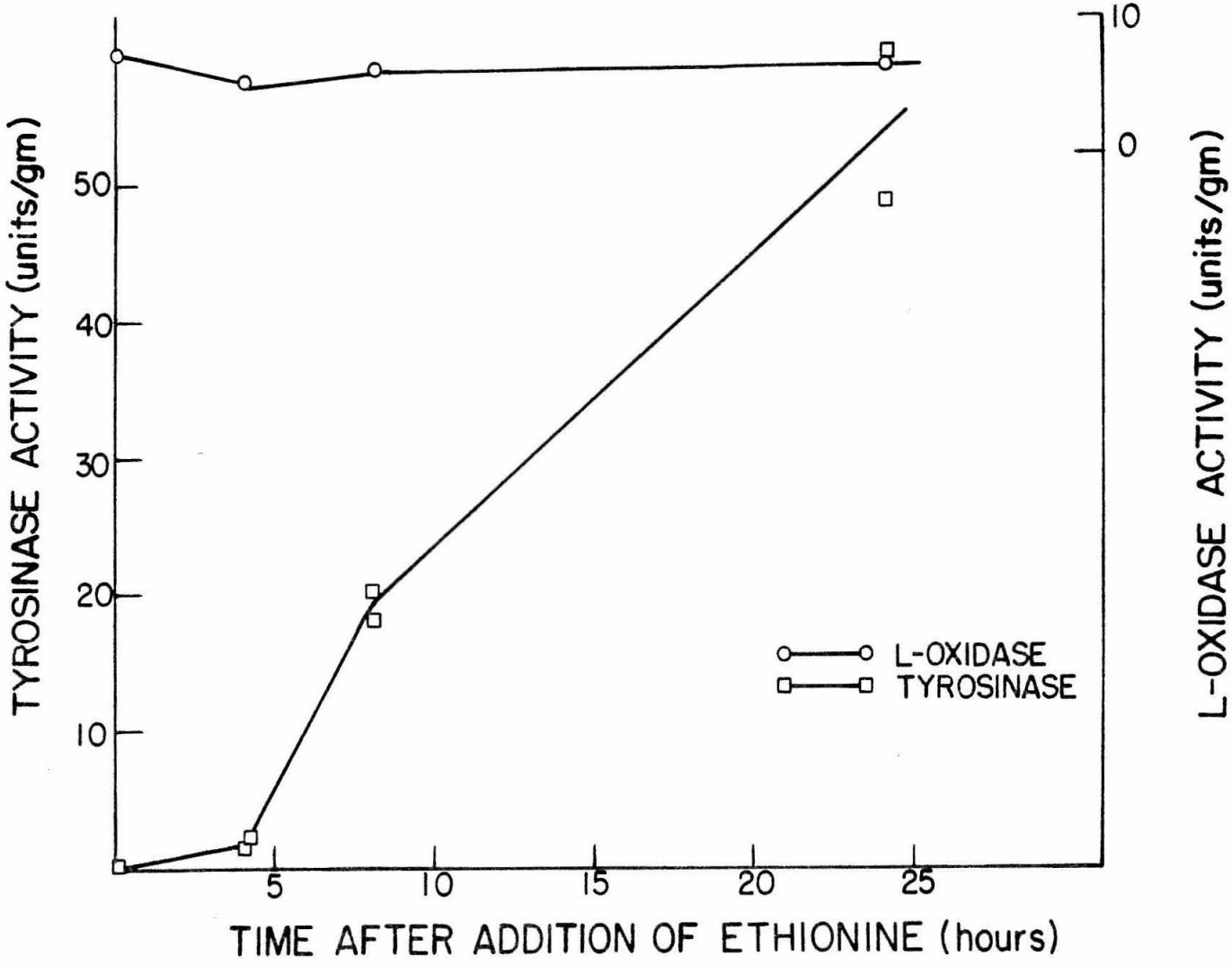
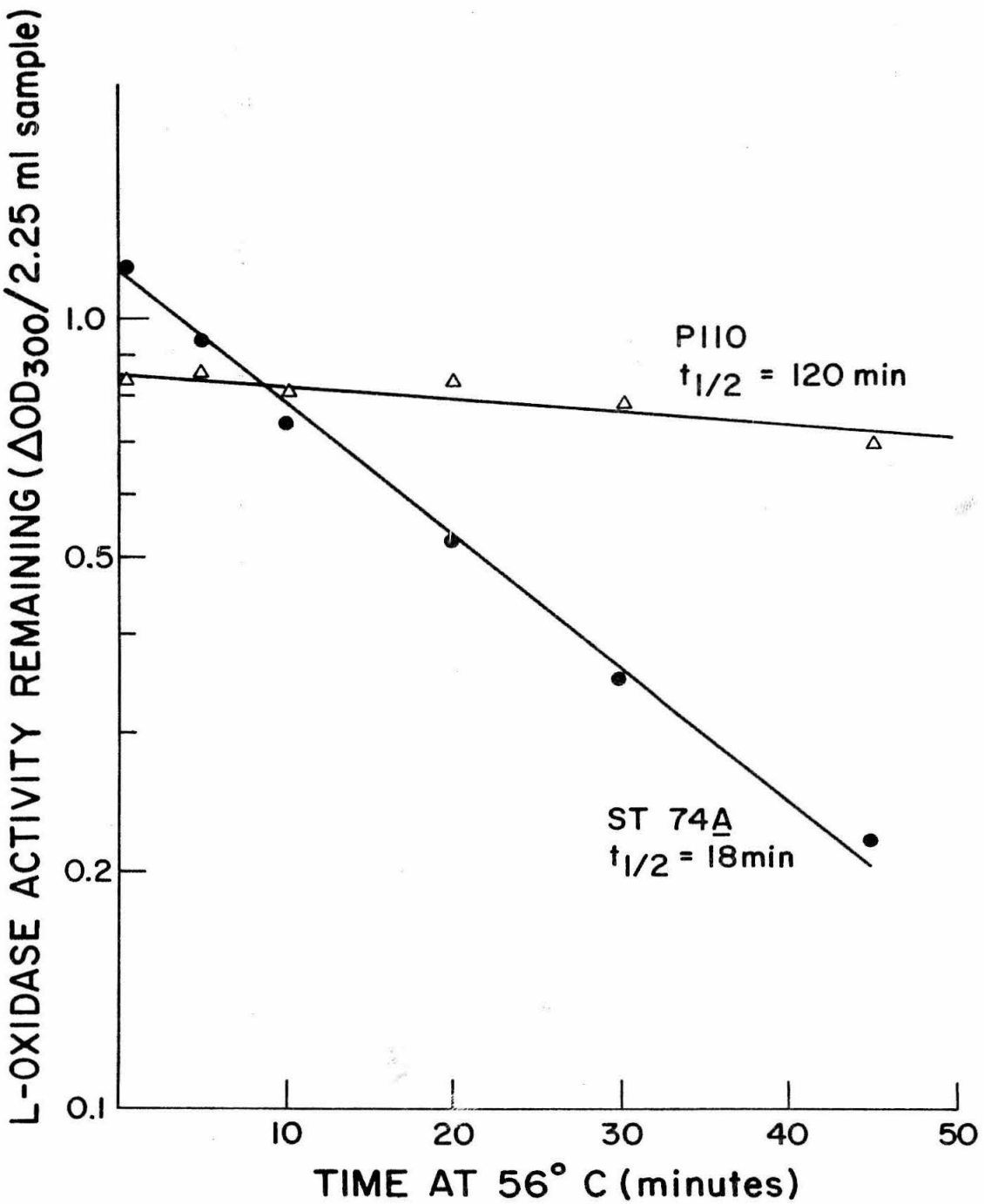

TYROSINASE ACTIVITY (units/gm)
50
40
30
20
10
10
0
L-OXIDASE ACTIVITY (units/gm)
L-OXIDASE
TYROSINASE
5
10
15
20
25
TIME AFTER ADDITION OF ETHIONINE (hours)

Figure 4. A comparison of the thermostability of crude L-amino acid oxidase prepared from derepressed wild-type and P110 grown on minimal medium. Stationary cultures of ST 74A were washed twice with 20 ml sterile distilled water and were then suspended on 5 ml sterile Vogel's salts containing 2.5 μg cycloheximide. Flasks were incubated for 48 hours at 25°C, without shaking and in the dark. P110 was grown without shaking at 25°C for 7 days.

To determine the thermostability of the L-amino acid oxidase, crude extract was added to 0.1M sodium phosphate buffer, pH 6, at 56°C. The solution was mixed thoroughly, and 2.25 ml samples were removed periodically. Samples were pipetted directly into beakers on ice. All samples were analyzed for L-oxidase activity as described in Materials and Methods.


L-OXIDASE ACTIVITY REMAINING (ΔOD300/2.25 ml sample)
1.0
0.5
0.2
0.1
P110
t1/2 = 120 min
ST 74A
t1/2 = 18min
10
20
30
40
50
TIME AT 56° C (minutes)

et al. (44) for purification of tyrosinase. The crude extracts were treated as outlined below:

(1) Nucleic acids were removed from the crude extract by precipitation with 2 per cent streptomycin sulfate.

(2) Protein was precipitated by 0.8 saturated ammonium sulfate. The precipitate was collected by centrifugation and suspended in buffer.

(3) Two volumes of cold (-10°C) acetone were added slowly to precipitate proteins. The precipitate was pelleted and the pellet extracted twice with buffer.

(4) Ammonium sulfate was added to 0.5-0.6 saturation. The precipitate was collected by centrifugation and suspended in buffer.

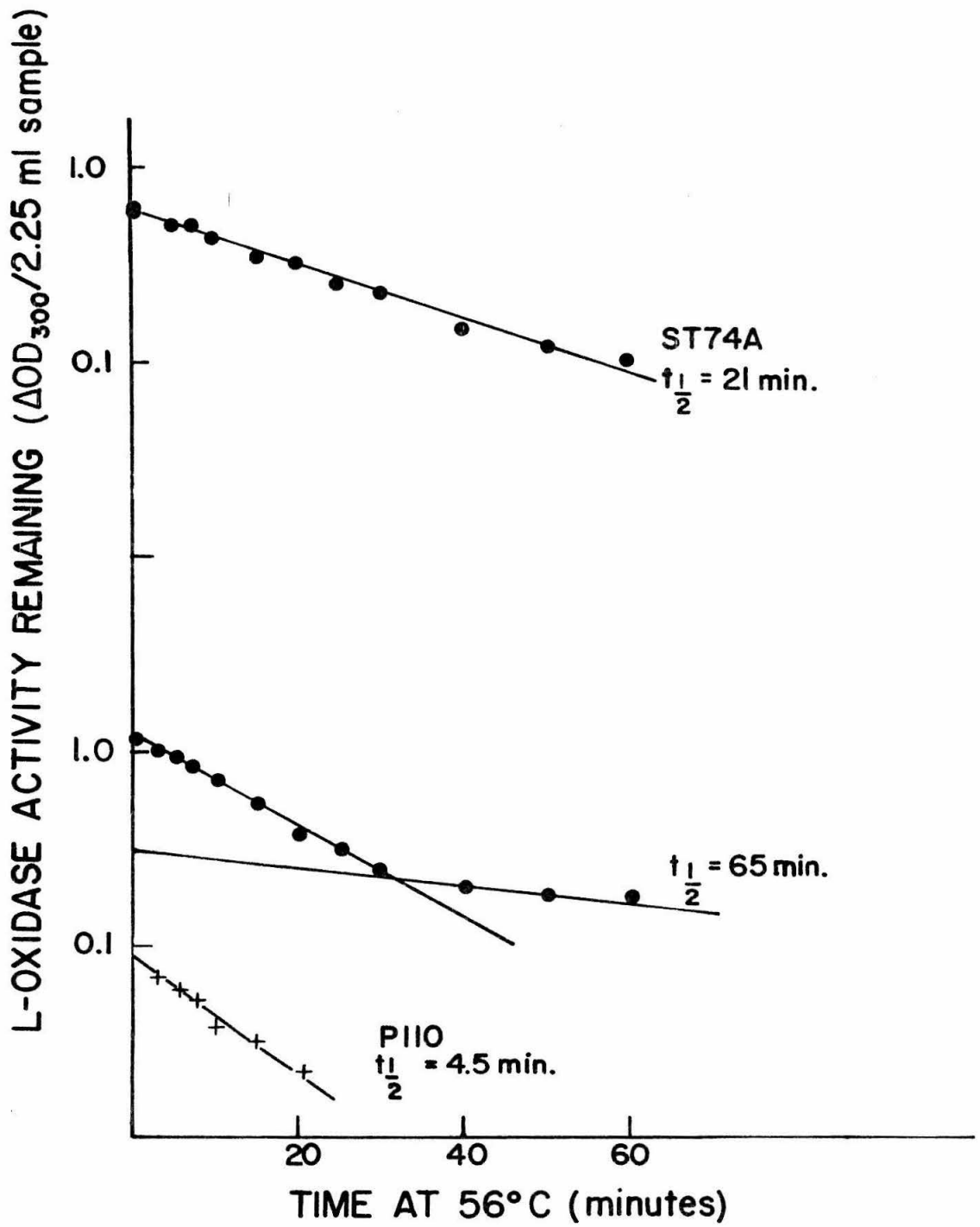
Results obtained with such partially purified preparations are shown in Figure 5. The oxidase from ST 74A did not show a significant change in thermostability upon purification, while the enzyme from P110 became more labile, and the denaturation curve appeared to be biphasic.

The steps indicated above resulted in a 2X increase in the specific activity of the oxidase from P110 and a 4.5X increase in the specific activity of the oxidase from ST 74A. Protein concentration was determined by the method of Lowry et al. (60). Major losses in L-amino acid oxidase activity were encountered during precipitation of the enzymes by cold acetone. The method published by Burton (3) for purification of the oxidase yielded a 4.5X increase in specific activity. To reduce losses in enzyme activity, and to improve the

Figure 5. A comparison of the thermostability of partially purified (method described in text) L-amino acid oxidase from derepressed wild-type and P110 grown on minimal medium. Stationary cultures of ST 74A were washed twice with 20 ml sterile distilled water and were then suspended on 5 ml sterile Vogel's salts containing 2.5 µg cycloheximide. Flasks were incubated for 48 hours at 25°C, without shaking and in the dark. P110 was grown without shaking at 25°C for 7 days.

To determine the thermostability of L-amino acid oxidase, 1.3 ml partially purified enzyme from ST 74A or 2.6 ml enzyme from P110 was added to 28 ml 0.1M sodium phosphate buffer, pH 6, at 56°C. Samples containing 2.25 ml heated enzyme were removed periodically and cooled in ice. All samples were analyzed for L-amino acid oxidase activity as described in Materials and Methods.

The thermostability of the more labile component in the extract was estimated after subtracting the activity due to the more stable component. The amount of activity due to the stable component present at early times during heat inactivation was estimated by extrapolating the curve of its decay to zero time.


L-OXIDASE ACTIVITY REMAINING (ΔOD300/2.25 ml sample)
1.0
0.1
1.0
0.1
ST74A
t1/2 = 21 min.
t1/2 = 65 min.
P110
t1/2 = 4.5 min.
20
40
60
TIME AT 56°C (minutes)

increase in specific activity, alternative methods of enzyme purification were investigated.

L-amino acid oxidase which had been partially purified as described above was found in the exclusion volume after gel filtration on Sephadex G 100, suggesting that the oxidase might be further purified by gel filtration on Sephadex G 200. The results of chromatography on Sephadex G 200 is shown in Figure 6. The oxidase is retarded slightly by G 200, and is eluted as a symmetrical peak of activity. Routinely, a 16-17X increase in specific activity was obtained with this simple procedure. Virtually all enzyme activity was recovered. L-oxidase from ST 74A which has been recovered from Sephadex G 200 has a thermostability which is nearly identical to the thermostability of similarly prepared oxidase from P110 (Figure 7). These results indicate that the mutation in P110 does not affect the structure of the L-amino acid oxidase produced by the mutant.

Both preparations show a biphasic denaturation curve. One explanation for the biphasic nature of the oxidase denaturation curve might be that the enzyme's cofactor, FAD, is rapidly lost upon heating. The early rapid loss of enzyme activity might represent the loss of FAD while the slower loss of activity might represent the destruction of the apoenzyme. Opposed to this suggestion was the finding that adding excess FAD to the solution during heating had no effect on the biphasic nature of the curve.

Figure 6. Elution profile of P110 L-amino acid oxidase chromatographed on Sephadex G 200. Part of the proteins in the crude extract were removed by adding solid ammonium sulfate to 30 per cent saturation at 4°C. The precipitate was spun down in a centrifuge and discarded. A 3 ml sample of the supernatant was applied to a G 200 column equilibrated with 0.1M sodium phosphate, pH 6. The column had a 4 cm zone of G 50 gel above the G 200 gel. The total column was 1.7 x 75 cm, and had an internal volume of 65-70 ml. L-amino acid oxidase was eluted with 0.1M sodium phosphate buffer, pH 6, using a pressure head of approximately 15 cm. Samples containing approximately 2 ml were collected automatically. Aliquots (0.4 ml) of each sample were assayed for L-amino acid oxidase. V_o was determined by chromatographing blue dextran 2000 (Pharmacia, Uppsala, Sweden) on the column.

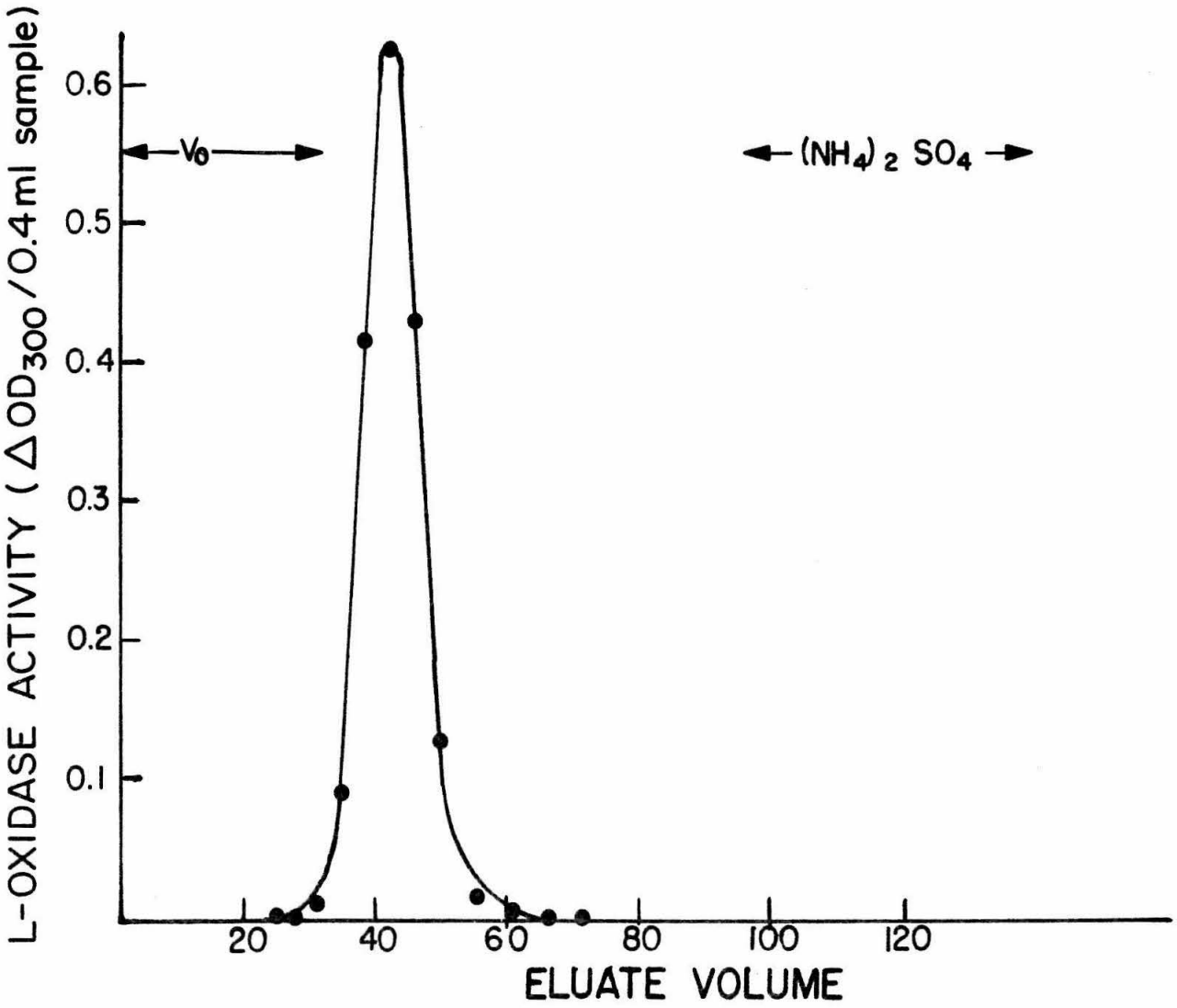
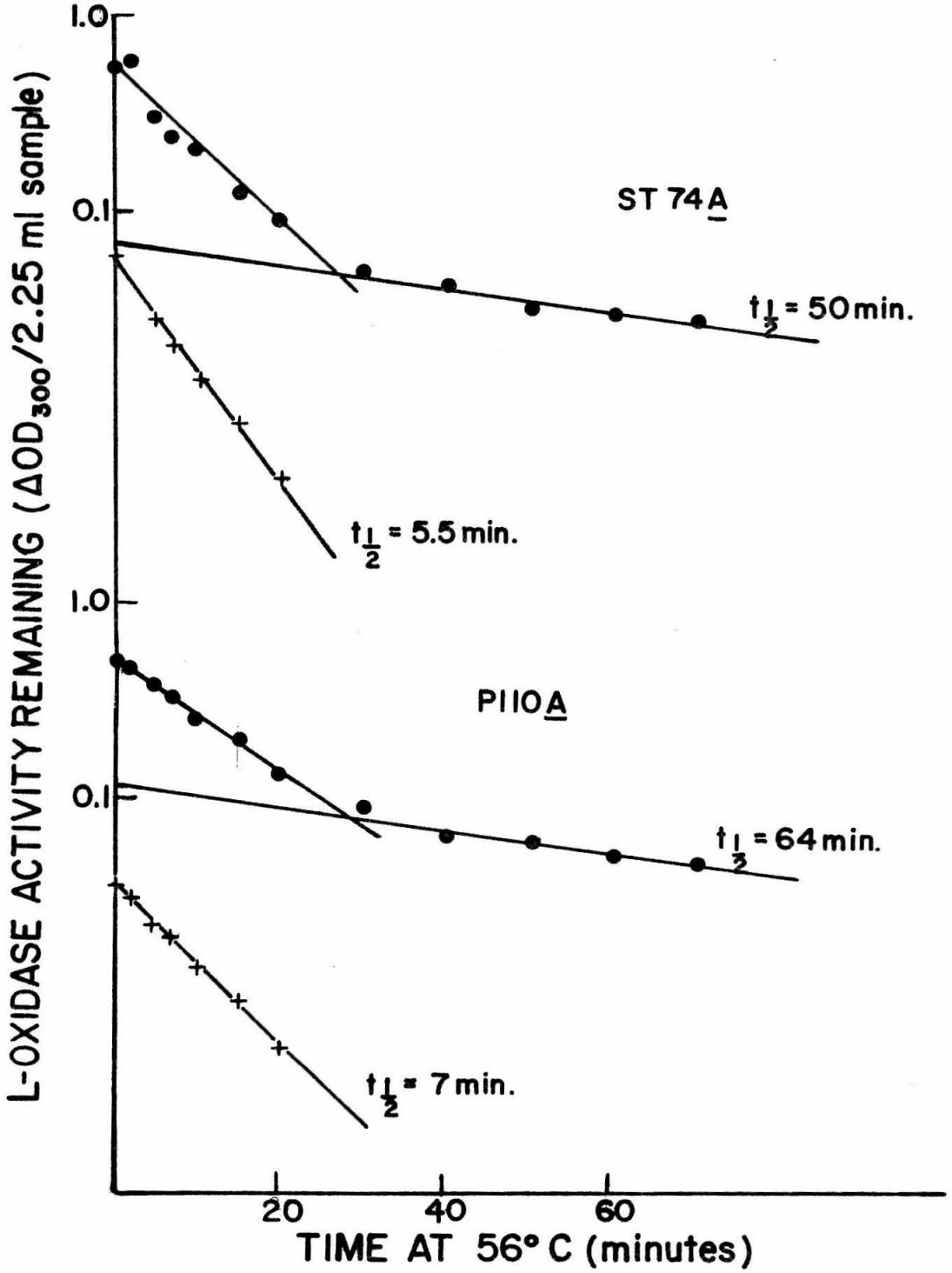

L-OXIDASE ACTIVITY (ΔOD300 /0.4 ml sample)
0.6
0.5
0.4
0.3
0.2
0.1
Vo
(NH4) 2 SO4
20
40
60
80
100
120
ELUATE VOLUME

Figure 7. A comparison of the thermostability of partially purified (using gel filtration) L-amino acid oxidase from derepressed wild-type and P110 grown on minimal medium. Stationary cultures of ST 74A were washed twice with 20 ml sterile distilled water and were then suspended on 5 ml sterile Vogel's salts containing 2.5 μg cycloheximide. Flasks were incubated for 48 hours at 25°C, without shaking and in the dark. P110 was grown without shaking at 25°C for 7 days.

To determine the thermostability of the L-amino acid oxidase, 5.2 ml of the combined fractions from G 200 which contained the L-oxidase peak were added to 24 ml 0.1M sodium phosphate buffer, pH 6, at 56°C. The solution was mixed thoroughly, and 2.25 ml samples were removed periodically. Samples were pipetted directly into beakers on ice. All samples were analyzed for L-oxidase activity as described in Materials and Methods.

The thermostability of the more labile component in the extract was estimated after subtracting the activity due to the more stable component. The amount of activity due to the stable component present at early times during heat inactivation was estimated by extrapolating the curve of its decay to zero time.


1.0
0.1
ST 74A
$t_{\frac{1}{2}}$ = 50 min.
$t_{\frac{1}{2}}$ = 5.5 min.
1.0
P110A
0.1
$t_{\frac{1}{2}}$ = 64 min.
$t_{\frac{1}{2}}$ = 7 min.
L-OXIDASE ACTIVITY REMAINING (ΔOD_{300}/2.25 ml sample)
20
40
60
TIME AT 56° C (minutes)

A Comparison of the L-Oxidase from Derepressed Wild-type and Induced Wild-Type

A second explanation for the biphasic nature of the oxidase denaturation curve could be that two oxidases are produced by derepressed cultures. This proposal was stimulated by a report by Sanwal and Lata that Neurospora produces two different glutamic acid dehydrogenases (61,62,63). In addition, I observed that L-amino acid oxidase, but not tyrosinase, is induced in wild-type cultures grown on L-α-amino-n-butyric acid as the sole nitrogen source in N-free Vogel's medium (the NH_4NO_3 in standard Vogel's salts was omitted; N-free trace elements were prepared by substituting $FeSO_4 \cdot 7\ H_2O$ for $Fe(NH_4)_2SO_4 \cdot 6H_2O$). Two per cent reagent grade sucrose was used in 20 ml medium in a 125 ml Erlenmeyer flask. Cultures were incubated without shaking at 25°C. A possible explanation for the synthesis of L-amino acid oxidase under these conditions could be that there are two L-amino acid oxidases, one of which is induced by growth on L-aminobutyric acid. Both L-oxidases could be derepressed by the conditions which derepress tyrosinase.

To determine whether cultures grown on aminobutyric acid as the sole nitrogen source produce an L-amino acid oxidase with properties which differ from those of the oxidase derepressed under conditions which also derepress tyrosinase, enzyme from the two sources was partially purified on G 200 and compared. The oxidase from cultures grown on aminobutyric acid was identical in size as determined by elution volume from the column (64) and thermostability to that from cycloheximide-derepressed cultures. The only difference noted in the

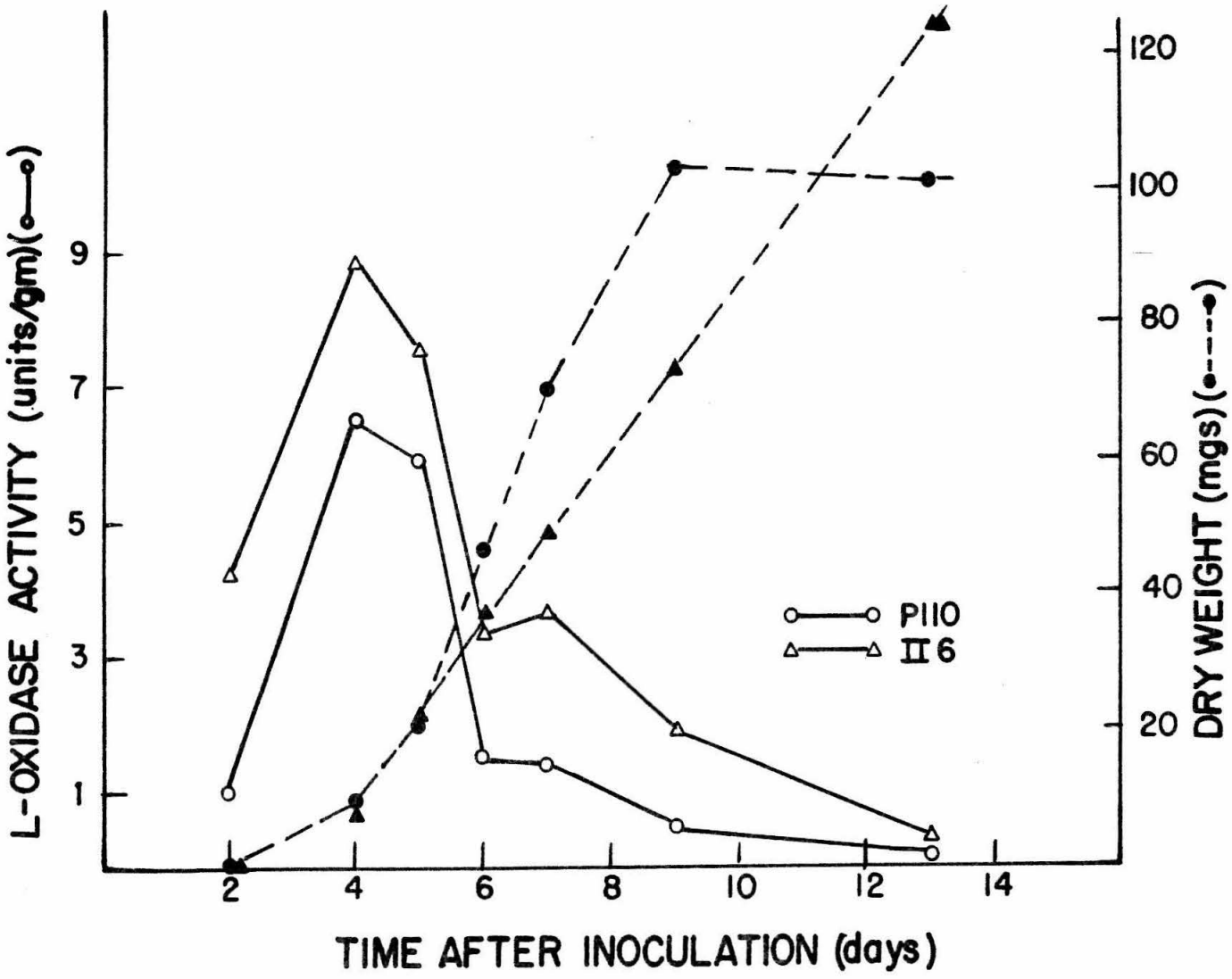
two preparations was a 2X higher specific activity in enzyme partially purified from aminobutyric acid-grown cultures. These results suggest that the same L-amino acid oxidase is formed in the two conditions studied.

The biphasic curve of heat inactivation observed with the L-oxidase may result from protection of the native enzyme by the denatured enzyme (65). Some support for this suggestion comes from the observed variability in the half-life of the more stable component (17.5 minutes - 61 minutes) contrasted to the reproducible half-life of the more labile component (3.2 minutes - 7 minutes).

Time Course of Growth and L-Oxidase Production by P110 and II6a;cot

Although the conidia of P110 contained no oxidase, 4 to 7 day old mycelial pads of P110 always contained a high level of the enzyme. To determine when the oxidase was synthesized during the growth of P110, the amount of oxidase present as a function of the age of the culture was investigated. A large number of 125 ml Erlenmeyer flasks containing 20 ml of minimal medium were inoculated concurrently. Groups of a few flasks were harvested at various times after inoculation and analyzed for dry weight or oxidase activity. The results are shown in Figure 8. Both P110A and II6a;cot showed a period of approximately 4 days during which very little growth occurred. After 4 days, growth proceeded at a rapid rate. The specific activity of L-amino acid oxidase was maximal at 4 days and decreased thereafter. The total oxidase activity per pad was observed to increase for 7 days.

Figure 8. Time course of growth and L-oxidase production by P110 and II6a;cot. Cultures were grown in 125 ml Erlenmeyer flasks containing 20 ml Vogel's minimal medium (40) and 2 per cent sucrose. Incubation was at 25°C, without shaking, for 2 to 13 days. Individual cultures were harvested at various times after inoculation and analyzed for dry weight or L-amino acid oxidase activity.


L-OXIDASE ACTIVITY (units/gm)(o—o)
DRY WEIGHT (mgs)(•---•)
TIME AFTER INOCULATION (days)
P110
II 6
1
3
5
7
9
20
40
60
80
100
120
2
4
6
8
10
12
14

Repression of the L-Oxidase Production of P110 by Keto Acids

The decrease in activity observed in older cultures suggested that the oxidase might be feedback repressed by keto acid products. This suggestion was tested by feeding II6a;cot filter-sterilized α-ketobutyrate, α-ketoglutarate, phenylpyruvate and pyruvate, each at a final concentration of 5 μmoles/ml. As shown in Table VI, each of the keto acids, with the exception of pyruvate, decreased the amount of oxidase observed in the cultures. The effect is particularly notable at pH 4.4, at which pH the keto acids are more readily taken up by the cultures than at pH 5.6. Aurich also investigated the effects of pyruvate on synthesis of L-oxidase and observed that pyruvate did not repress the enzyme (66). The effects of other keto acids were not investigated by him. Neither ketobutyrate nor ketoglutarate significantly inhibits the oxidase reaction, as shown in Table VII, suggesting that the keto acids repress the synthesis of the oxidase. Phenylpyruvate is the product of the reaction used to assay the oxidase, and thus was not tested as an inhibitor of the reaction.

The Effects of Keto Acids on Derepressed Wild-type Cultures

The finding that keto acids repress the synthesis of L-oxidase by II6a;cot led to an investigation of the effects of keto acids on derepressed cultures. In wild-type cultures derepressed by the addition of ethionine (41), keto acids repressed the synthesis of tyrosinase as well as L-oxidase (Table VIII). That the keto acid did not act by preventing the uptake of ethionine was shown by adding the keto acid 1 to

TABLE VI

Repression of L-amino acid oxidase synthesis by keto acids

Keto acid added	pH	Dry wt (mg) -5 days	Oxidase activity (ECU/gm)
none	5.65	15.1	9.5
ketobutyrate	5.65	24.1	2.1 (78%)*
ketoglutarate	5.65	15.5	3.8 (60%)
phenylpyruvate	5.65	28.0	4.0 (58%)
pyruvate	5.65	16.3	8.6 (9.5%)
none	4.4	12.0	2.7
ketobutyrate	4.4	15.5	0.17 (94%)
ketoglutarate	4.4	13.0	0.30 (89%)
phenylpyruvate	4.4	23.3	0.4 (85%)
pyruvate	4.4	12.1	3.2 (0%)

*Per cent inhibition. Cultures were grown in 20 ml Vogel's minimal medium in 125 ml Erlenmeyer flasks standing at 25°C. Adjustment of the pH was made prior to autoclaving the flasks. Filter-sterilized keto acids (5 μmole/ml) were added after flasks were autoclaved.

TABLE VII

The effect of keto acids on the L-oxidase reaction

Additions to the reaction mixture	L-oxidase activity (ECU/gm)
none	2.1
10 μmoles ketoglutarate	1.9 (9.5%)*
none	3.7
11 μmoles ketobutyrate	3.5 (5.4%)

*Per cent inhibition.

TABLE VIII

The effect of keto acids on synthesis of L-oxidase and tyrosinase by ethionine-derepressed ST 74A

Conditions	L-oxidase (ECU/gm)	Tyrosinase (ECU/gm)
Control. No ethionine added	0.067	1.7
Ethionine added	11.2	270
Ethionine and phenylpyruvate added simultaneously	2.3 (79%)*	89 (67%)*
Phenylpyruvate added 1 hour after ethionine	2.3 (79%)*	76 (72%)*
Control. No ethionine added	0.013	0
Ethionine added	5.0	144
Ethionine and ketobutyrate added simultaneously	0.92 (82%)*	33 (77%)*
Ketobutyrate added 2 hours after ethionine	2.5 (50%)*	96 (33%)*

*Per cent decrease in activity. Cultures were grown for 2 days on 1/2 X Vogel's salts and 1/2% sucrose, 20 ml medium per 125 ml Erlenmeyer flask. Cultures were incubated without shaking at 25°C. DL-ethionine was added to give a final concentration of 100 μg/ml. Filter-sterilized phenylpyruvate was added to give a final concentration of 1 mg/ml; ketobutyrate was added to give a final concentration of 0.5 mg/ml. After addition of ethionine to the test cultures, all cultures were incubated on a reciprocal shaker at 25°C for an additional 2 days before harvesting.

2 hours after the addition of ethionine. By this time, most of the ethionine would have been assimilated by the culture (41).

The addition of keto acids to cycloheximide-derepressed wild-type cultures did not affect the production of either tyrosinase or L-amino acid oxidase, as shown in Table IX. In the experiment using ketobutyrate, the result might be explained by inhibition of keto acid uptake by cycloheximide, but in the experiment using phenylpyruvate, the keto acid was added prior to the addition of cycloheximide.

A plausible explanation for the difference between the effect of keto acids on the two antimetabolites could be that keto acids somehow influence the level of methionine in the cell. Keto acids might inhibit the activity of amino acid degrading enzymes by end product inhibition, for example. If the intracellular concentration of methionine were increased, one would expect a reduction in the effectiveness of ethionine, whereas an increase in methionine would not be expected to influence derepression by cycloheximide.

Whether the effects of keto acids on ethionine-derepressed wild-type cultures is related to the effects of keto acids on the mutant is not clear. In derepressed cultures, the keto acid effects are not specific to production of the L-oxidase.

Identification of a Factor from Yeast Extract Which Improves the Growth of P110

The poor growth of P110 and all its progeny which synthesize oxidase constitutively on minimal medium has been mentioned previously.

TABLE IX

The lack of an effect of keto acids on synthesis of L-oxidase and tyrosinase by cycloheximide-derepressed ST 74A

Conditions	L-oxidase (ECU/gm)	Tyrosinase (ECU/gm)
Control. No cycloheximide added	0.013	0
Cycloheximide added	3.18	143
Cycloheximide and ketobutyrate added simultaneously	3.54	137 (4.2%)*
Ketobutyrate added 2 hours after cycloheximide	4.43	155
Control. No cycloheximide added	0.067	1.7
Cycloheximide added	4.44	517
Phenylpyruvate added 1 hour before cycloheximide	7.9	409 (21%)*
Cycloheximide and phenylpyruvate added simultaneously	9.6	438 (15%)*

*Per cent decrease in activity. Cultures were grown for 2 days on 1/2 X Vogel's salts and 1/2% sucrose, 20 ml medium per 125 ml Erlenmeyer flask. Cultures were incubated without shaking at 25°C. Cycloheximide was added to give a final concentration of 0.2 μg/ml. Filter-sterilized phenylpyruvate was added to give a final concentration of 1 mg/ml; ketobutyrate was added to give a final concentration of 0.5 mg/ml. After addition of cycloheximide to the test cultures, all cultures were incubated at 25°C on a reciprocal shaker for an additional 2 days before harvesting.

Growth of the cultures is improved on complete medium, which in addition represses synthesis of the oxidase (7). The enhancement of growth of the mutant by complete medium and the shape of the growth curve on minimal medium (Figure 8) suggested that P110 was an incompletely blocked auxotroph. Evidence in support of this suggestion came from the following experiment. Mycelium from II6a;cot grown on minimal medium was extracted in boiling water immediately after harvesting. The boiling water extract was added to minimal medium which was then inoculated with a fresh conidial suspension of II6a;cot. The mutant grew significantly better in the presence of the extract than on minimal medium. The results suggested that the compound required by II6a;cot was slowly synthesized and accumulated by the mutant. The compound required for good growth of P110 and its progeny was postulated to be a repressor or co-repressor of L-oxidase synthesis. As the supply of this compound increased in the mutant, L-oxidase activity would decrease, as was observed in the experiments measuring enzyme activity as a function of the age of the culture.

Keto acids had been found to repress the synthesis of oxidase, but none of those tried had improved the growth of P110 nearly as much as complete medium. The individual components of complete medium were tested for their effects on the growth of P110. As shown in Table X, the only single component which mimicked complete medium was yeast extract. Hydrolyzed casein did not support good growth of the mutant. Auxanographic tests with a large number of individual vitamins and

TABLE X

The effects of the components of complete medium on the growth of P110

Components added to minimal medium	ST 74A dry wt (mg)	P110 dry wt (mg)
none	33.4	trace
5 mg/ml yeast extract	54.3	23.5
0.25 mg/ml casein hydrolysate	43.0	trace
5 mg/ml malt extract	37.7	trace
yeast extract and casamino acids	48.5	18.5
malt extract and casamino acids	36.9	15.3
yeast extract and malt extract	47.3	23.3
yeast extract, malt extract, and casamino acids	41.1	18.6

Cultures were grown for 2 days in standing cultures containing 20 ml medium in 125 ml Erlenmeyer flasks. Cultures were incubated at 25°C.

amino acids gave negative results. Thus yeast extract was fractionated to isolate the compound required by P110.

A component of yeast extract which improved the growth of P110 was isolated as follows. Twenty grams of yeast extract were dissolved in 100 ml distilled water. Absolute ethanol (900 ml) was added with stirring. The precipitate was pelleted by centrifugation and discarded. Ethanol in the supernatant was removed under reduced pressure in a Calab Model C Evaporator. The residue was dissolved in distilled water, and the pH adjusted to 2 with concentrated HCl. Organic acids and lipids were removed by 3 extractions with ether and the ether phases were discarded. The water phase from the ether extraction was adjusted to pH 7. Dowex 1-X1 was added to adsorb anions. The eluate from Dowex 1 was neutralized and adsorbed with Dowex 50W-X4 to remove cations. The yellow solution was neutralized and then adsorbed with charcoal. The clear and colorless solution was concentrated 10X, and 5 ml were chromatographed on Biogel P2 (3 cm x 58 cm). The active compound was eluted with water at V_e/V_o = 1.81, indicating a molecular weight of approximately 200. The size of the molecule and the fact that it was uncharged suggested that it might be a sugar. None of the following sugars at concentrations of 2 per cent improved the growth of P110, either alone or in combination with sucrose (also at 2 per cent): lactose, mannitol, fructose, glucose, lyxose, L- or D-arabinose, raffinose, rhamnose, maltose, galactose, ribose, xylose, or dextrin.

The fact that the component in complete medium affected synthesis of L-amino acid oxidase suggested that the factor might be an amino acid or an amino acid analogue. The studies of Thayer and Horowitz (2) and Burton (3) suggested that the L-amino acid oxidase could be induced by substrate amino acids. Studies concerning induction and repression of enzymes involved in arginine biosynthesis indicate that a structural analogue of the repressor may specifically reverse repression (i.e. "induce") (76). In the case of P110, it seemed possible that non-substrate amino acids or an amino acid analogue might act as a repressor. The proteinogenic amino acids had been eliminated by tests with casamino acids and auxanographic tests. The amino acids in the most active fraction from the P2 gel were separated by high voltage electrophoresis. No unusual amino acids were present, but alanine, glycine, serine, threonine, methionine, and phenylalanine were present. Each of these amino acids was tested individually and in combination with the others. The results shown in Table XI indicated that serine or glycine enhanced the growth of P110, and that the combination of amino acids was especially stimulatory. From these results P110 appeared to be an incompletely blocked auxotroph requiring serine or glycine. A review of the literature indicated that other serineless mutants of Neurospora do not utilize casein hydrolysate (67,68,69). As shown in later experiments, P110 requires more serine for growth than is provided by the concentration of casein hydrolysate added as supplement. Why the auxanographic tests gave negative results is unknown.

TABLE XI

The effect on growth of P110 of the amino acids present in the active fraction of yeast extract

Supplement (4 mg/flask)	Dry wt in mg
none	2.5
alanine	2.9
glycine	18.4
methionine	5.2
phenylalanine	3.2
serine	26.4
threonine	2.2
All 6 amino acids above	55.0

Cultures were grown for 3 days in standing cultures containing 20 ml medium in 125 ml Erlenmeyer flasks. Cultures were incubated at 25°C.

Studies Concerning the Amino Acid Requirement of P110 and Its Relationship to L-Amino Acid Oxidase Synthesis

The Effect of Inoculum Size on Utilization of Glycine by P110

To confirm that serine and/or glycine was the compound(s) required by P110, 17 amino acids were tested individually. The results, shown in Table XII, indicated that serine was the only single amino acid which markedly improved the growth of P110 or II6a;cot. In this experiment, glycine did not appear to be equivalent to serine, whereas it had given good growth in earlier studies. Hungate reported (67) a similar variability in the response to glycine by H 605 ser-1, but gave no explanation for it. The inoculum size was not controlled in these experiments, nor in those reported by Hungate. Figure 9 indicates that a 10-fold variation in the number of conidia used for inoculation makes a significant difference in the amount of growth seen on glycine. Conidial suspensions prepared in the manner routinely used in growth experiments could easily vary by a factor of ten in concentration. Growth of the mutant on serine or minimal medium shows a similar, but much less marked, effect.

Conversion of Glycine to Serine in vivo

Evidence that P110 could convert glycine to serine was obtained from labeling studies. Each culture was grown on unsupplemented Vogel's minimal medium until a mycelial pad of convenient size had formed. P110 was grown for 6 days; ST 74A was grown for 3 days. Each pad was washed

TABLE XII

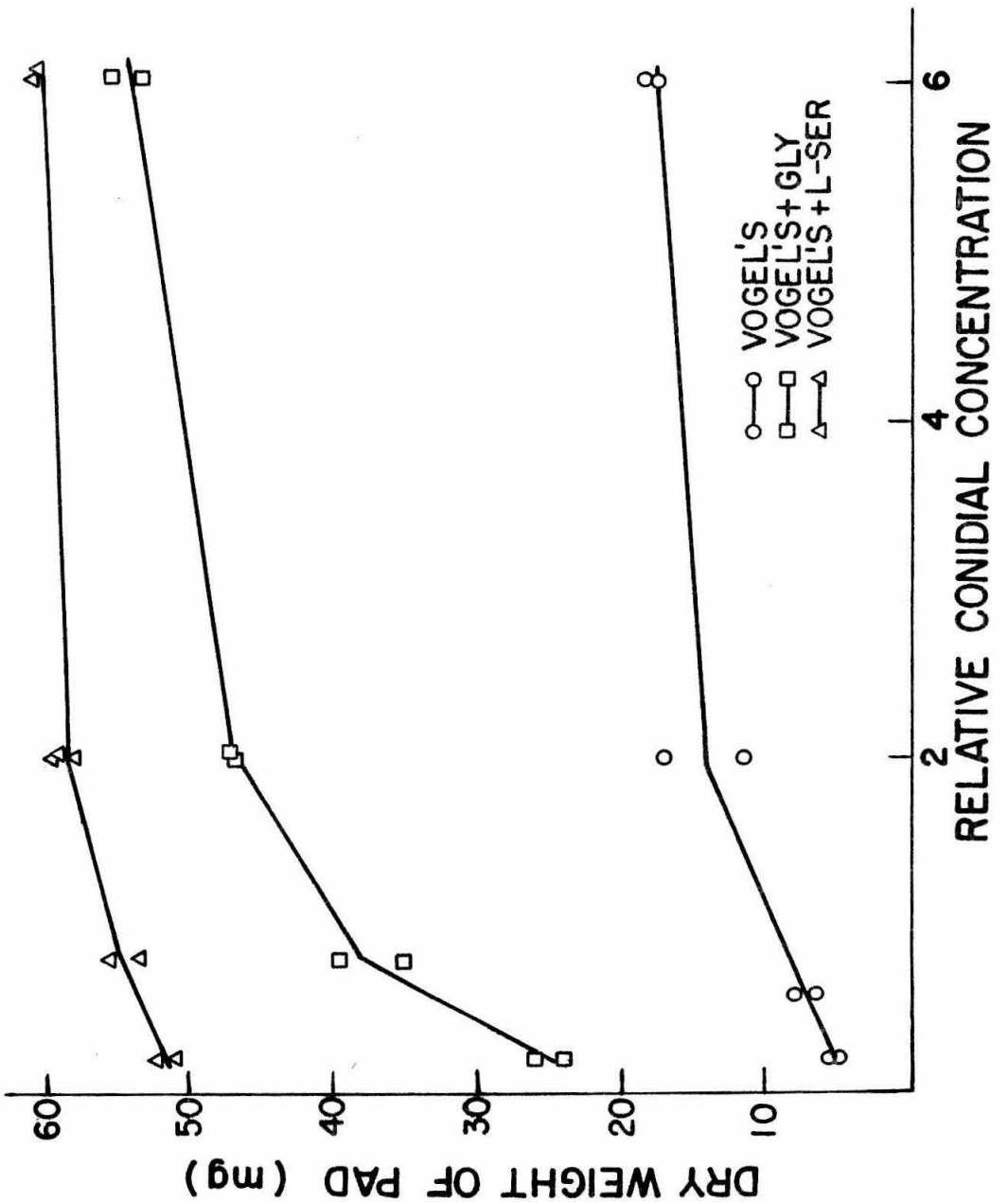
The effect of individual amino acids on the growth of P110, II6 and ST 74A

Added amino acid	P110	II6	ST 74A
	mg dry wt	mg dry wt	mg dry wt
none	2.0	2.4	55.8
DL-alanine	1.3	2.0	59.8
L-arginine	1.4	2.9	67.4
L-aspartic acid	1.9	2.6	63.0
L-cysteine	trace	2.2	16.0
L-glutamic acid	1.7	2.3	60.8
glycine	6.4	3.8	39.7
L-histidine	1.3	2.3	52.8
L-isoleucine + L-valine	2.4	1.9	60.2
L-leucine	1.7	1.9	62.0
L-lysine	1.1	2.3	63.6
L-methionine	4.7	4.1	61.6
L-phenylalanine	3.0	2.1	57.2
L-proline	1.8	2.4	63.7
L-serine	27.8	45.4	63.7
DL-threonine	1.6	1.8	62.8
L-tryptophan	6.2	3.0	58.1
L-tyrosine	2.9	2.6	86.9
L-serine + glycine + L-methionine	54.9	63.5	70.1
L-serine + glycine + L-methionine + L-tryptophan	56.3	51.9	68.2

TABLE XII (continued)

Cultures were grown for 3 days in 20 ml Vogel's minimal medium in 125 ml Erlenmeyer flasks at 25°C, without shaking. Amino acids were added to give a final concentration of 0.2 mg/ml, with the exception of DL-alanine, which was tested at 0.4 mg/ml. All dry weights are averages of duplicate pads.

Figure 9. The effect of inoculum size on utilization of glycine by P110. Cultures were grown in 125 ml Erlenmeyer flasks containing 20 ml Vogel's medium and 2 per cent sucrose. Glycine or serine was added to give a final concentration of 5 μmoles/ml. The conidial suspension of P110 was diluted serially by factors of ten and 2 or 6 drops of each dilution were used to inoculate duplicate flasks of each medium. Cultures were grown without shaking at 25°C for 96 hours.

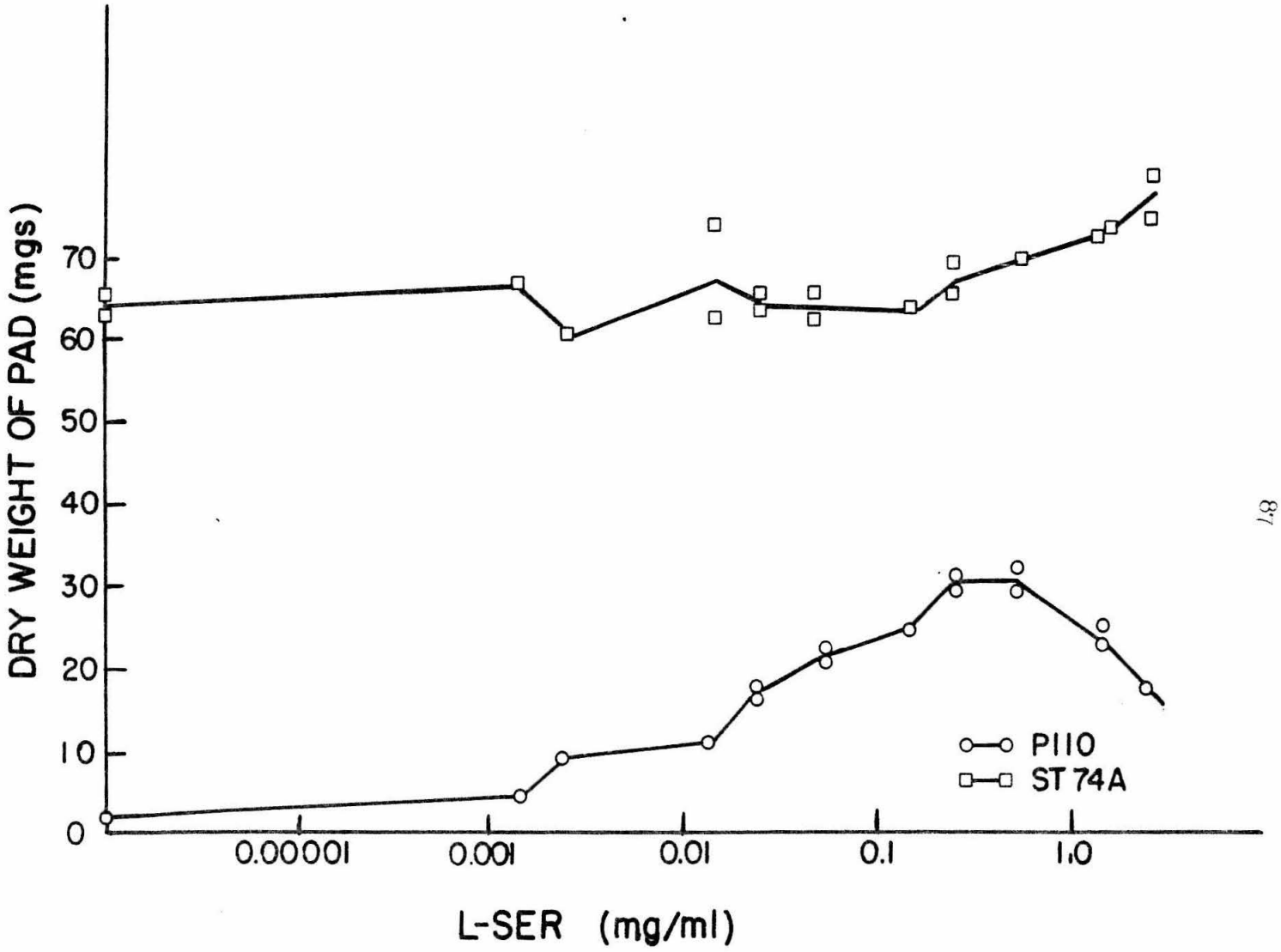

DRY WEIGHT OF PAD (mg)
60
50
40
30
20
10
2
4
6
RELATIVE CONIDIAL CONCENTRATION
VOGEL'S
VOGEL'S+GLY
VOGEL'S+L-SER

twice with 20 ml sterile distilled water and suspended on 10 ml fresh Vogel's medium containing 2 per cent sucrose. Cultures were equilibrated for 45 minutes at 25°C with shaking. Ten ml of Vogel's medium containing 2 per cent sucrose and 0.5 μc (5 μmoles) C^{14}-U-glycine were added to each culture. Incubation was continued on the shaker at 25°C. Cultures were harvested 10 minutes or 30 minutes after the addition of labeled glycine. The intracellular free amino acid pools were extracted immediately and the amino acids were separated by high voltage electrophoresis. Radioactivity was observed in just two amino acids, serine and glycine. Pl10 showed a greater fraction of the counts in serine at both 10 minutes and 30 minutes than did ST 74A, although both cultures clearly converted glycine to serine.

Growth of Pl10 as a Function of Serine Concentration

As shown in Table XII, serine at 0.2 mg/ml did not allow Pl10 to grow as well as wild-type, nor was it as effective in supporting growth as a combination of serine, glycine and methionine. These results suggested that Pl10 might require more than 0.2 mg/ml L-serine for optimum growth. The results of a concentration series shown in Figure 10 indicate that optimum growth of Pl10 is obtained at 0.25-0.5 mg/ml L-serine and that higher concentrations are somewhat inhibitory. No concentration of serine allowed the mutant to grow more than one-half as much as the wild-type within 3 days at 25°C in standing cultures. The high concentration of serine required by the mutant may explain why hydrolyzed casein does not support the growth of Pl10.

Figure 10. Growth of P110 and ST 74A as a function of L-serine concentration. Cultures were grown in 125 ml Erlenmeyer flasks containing 20 ml Vogel's medium and 2 per cent sucrose. L-serine was added at concentrations ranging from 0.5 μg/ml to 5.25 mg/ml. Flasks were incubated at 25°C without shaking for 3 days.


DRY WEIGHT OF PAD (mgs)
70
60
50
40
30
20
10
0
0.00001
0.001
0.01
0.1
1.0
L-SER (mg/ml)
P110
ST 74A

Hydrolyzed casein was fed at a concentration of 0.25 mg/ml, which would supply much less than the required amount of L-serine. Casein contains 5.4-6.7 g serine per 16 g amino nitrogen (70).

Repression of L-Oxidase Synthesis in P110 by Serine and Related Metabolites

In addition to their effect on growth, both complete medium and yeast extract repressed the synthesis of L-amino acid oxidase by P110. Once serine had been identified as the growth-promoting factor in yeast extract, its effect on synthesis of L-amino acid oxidase by P110 was investigated. Because metabolites related to serine seemed to have an added effect on the growth of the mutant, their effects on enzyme synthesis were studied as well. At 0.1 mg/ml, serine reduced the amount of L-oxidase observed in the mutant, but combinations of serine and its related metabolites were more effective than serine alone, as shown in Table XIII. In particular, tryptophan in combination with any other two supplements significantly reduced the level of oxidase produced by P110. Adding yeast extract to the metabolites related to serine did not further reduce the amount of oxidase produced by P110, but did improve the growth of the mutant. Growth equal to that obtained on yeast extract was obtained by adding asparagine to the combination of compounds related to serine.

TABLE XIII

The effects of serine and related metabolites on growth and L-oxidase in P110

Additions to Vogel's medium	Dry wt (mg)	L-oxidase (ECU/gm)
none	4.3	6.0
serine	33.6	4.0
serine + glycine	45.0	2.1
serine + adenine	32.0	3.2
serine + methionine	44.0	1.8
serine + tryptophan	36.3	2.0
serine + yeast extract	92.4	0.22
ser + gly + met	60.5	1.3
ser + gly + adenine	47.6	1.1
ser + gly + tryp	56.2	0.49
ser + met + tryp	48.0	0.25
ser + tryp + adenine	42.0	0.58
ser + met + adenine	49.2	1.6
ser + gly + met + tryp + ad	65.3	0.13
5 above + yeast extract	86.1	0.13
5 above + asparagine	89.0	----

Cultures were grown for 3 days without shaking at 25°C in 20 ml medium in 125 ml Erlenmeyer flasks. All values given in the table are averages of duplicate cultures. Serine was supplied at 0.1 mg/ml; all other supplements were added to give a final concentration of each of 0.2 mg/ml.

The Effects of Serine and Glycine on Growth and L-Oxidase Production by P110 as a Function of Time

The variation in oxidase activity as a function of the age of the culture has been discussed previously. It was of interest to determine what effect serine or glycine would have on the growth and enzyme production of P110 as a function of time. The results shown in Figure 11 indicated that in this experiment serine and glycine were equal in their ability to stimulate early growth of the culture. The lag period on serine or glycine was reduced to 2 days, and thereafter, the growth rate was equivalent to that seen on minimal medium after 4 days. By 7 days, P110 had grown as well on minimal medium as it had on medium supplemented with serine. Glycine allowed P110 to grow to a final dry weight equal to that of the wild-type control (see Figure 12), but optimum growth was reached after 8 days in the case of the mutant, compared to 6 days in the case of the wild-type. Glycine, but not serine, was inhibitory to the germination or early growth of wild-type ST 74A.

Despite the similarity in their effects on the growth of P110, serine and glycine differed in their effects on synthesis of oxidase by the mutant. In Figure 13, serine is shown to decrease the rate of enzyme synthesis per mg dry weight of the mutant, while glycine has little effect. When added to the reaction mixture used to assay L-oxidase, serine and glycine were only slightly inhibitory, as shown in Table XIV.

Figure 11. Time course of growth of P110 on minimal medium compared to growth on L-serine or glycine. Cultures were grown on 20 ml Vogel's medium and 2 per cent sucrose in 125 ml Erlenmeyer flasks. Glycine or serine was added to give a final concentration of 5 μmoles/ml. Flasks were incubated without shaking at 25°C. Duplicate cultures were harvested and their dry weights determined at 2 to 11 days after inoculation.

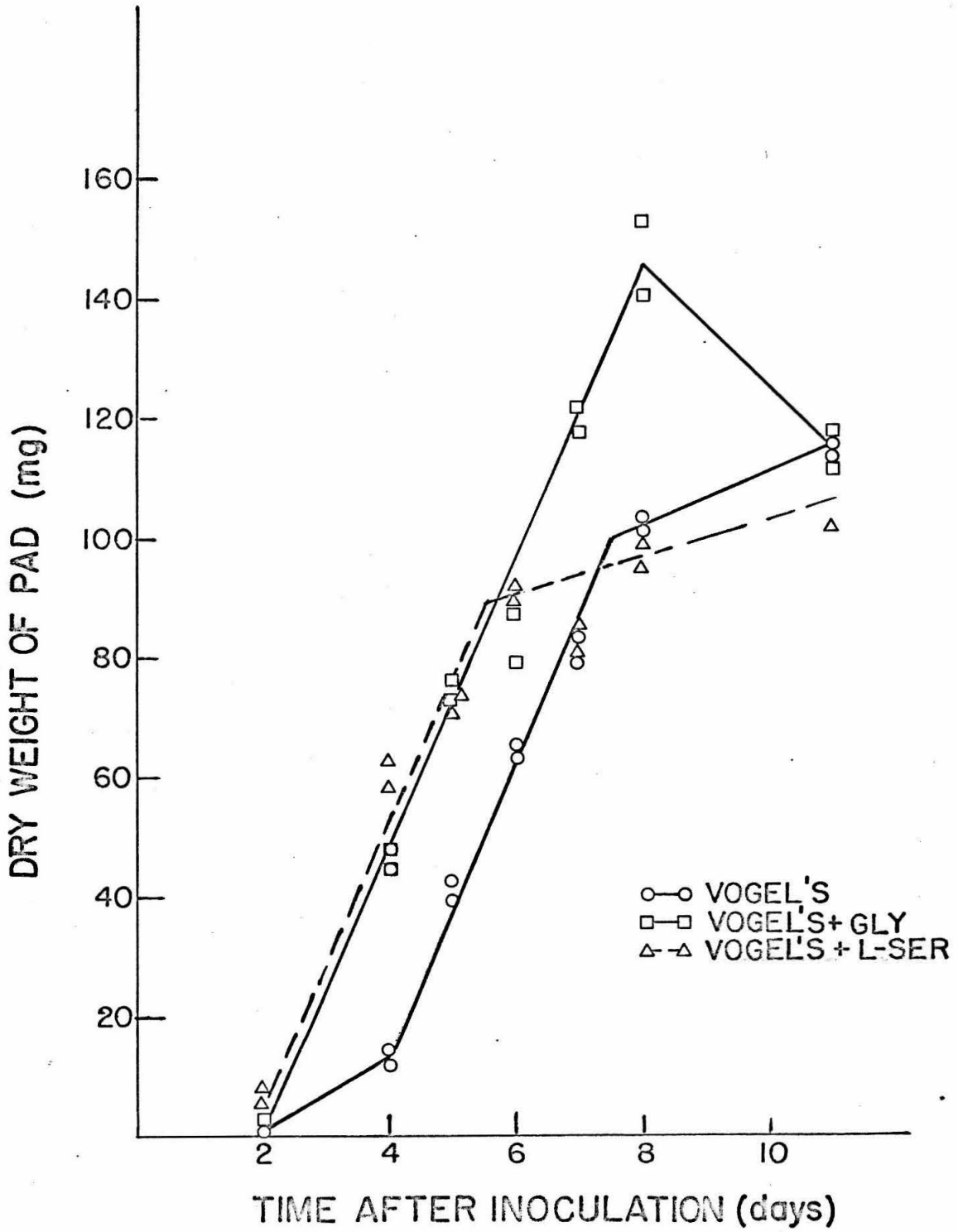

DRY WEIGHT OF PAD (mg)
160
140
120
100
80
60
40
20
2
4
6
8
10
TIME AFTER INOCULATION (days)
VOGEL'S
VOGEL'S+ GLY
VOGEL'S + L-SER

Figure 12. Time course of growth of ST 74A on minimal medium compared to growth on L-serine or glycine. Cultures were grown on 20 ml Vogel's medium and 2 per cent sucrose in 125 ml Erlenmeyer flasks. Glycine or serine was added to give a final concentration of 5 μmoles/ml. Flasks were incubated without shaking at 25°C. Duplicate cultures were harvested and their dry weights determined at 2 to 11 days after inoculation.

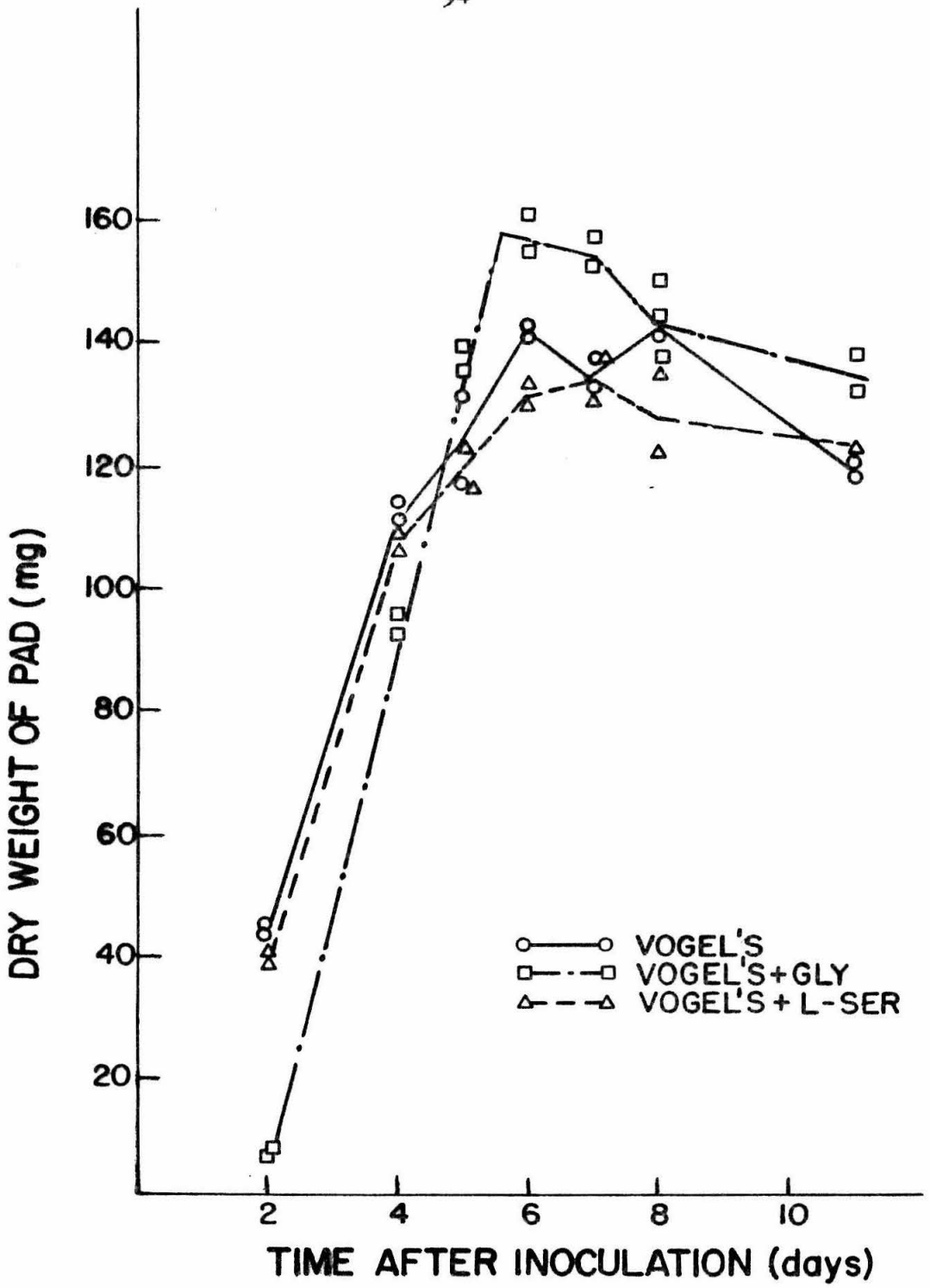

DRY WEIGHT OF PAD (mg)
160
140
120
100
80
60
40
20
2
4
6
8
10
TIME AFTER INOCULATION (days)
VOGEL'S
VOGEL'S+GLY
VOGEL'S + L-SER

Figure 13. The effect of L-serine and glycine on the rate of L-amino acid oxidase synthesis in P110. Cultures were grown on 20 ml Vogel's medium and 2 per cent sucrose in 125 ml Erlenmeyer flasks. Glycine or serine was added to give a final concentration of 5 μmoles/ml. Flasks were incubated without shaking at 25°C. Duplicate cultures were harvested and assayed for L-amino acid oxidase at various times after inoculation. Dry weights were determined on separate pads harvested simultaneously. Each point on the graph is the average value of two dry weights and the average value of two L-oxidase activities. All duplicate values were in close agreement.

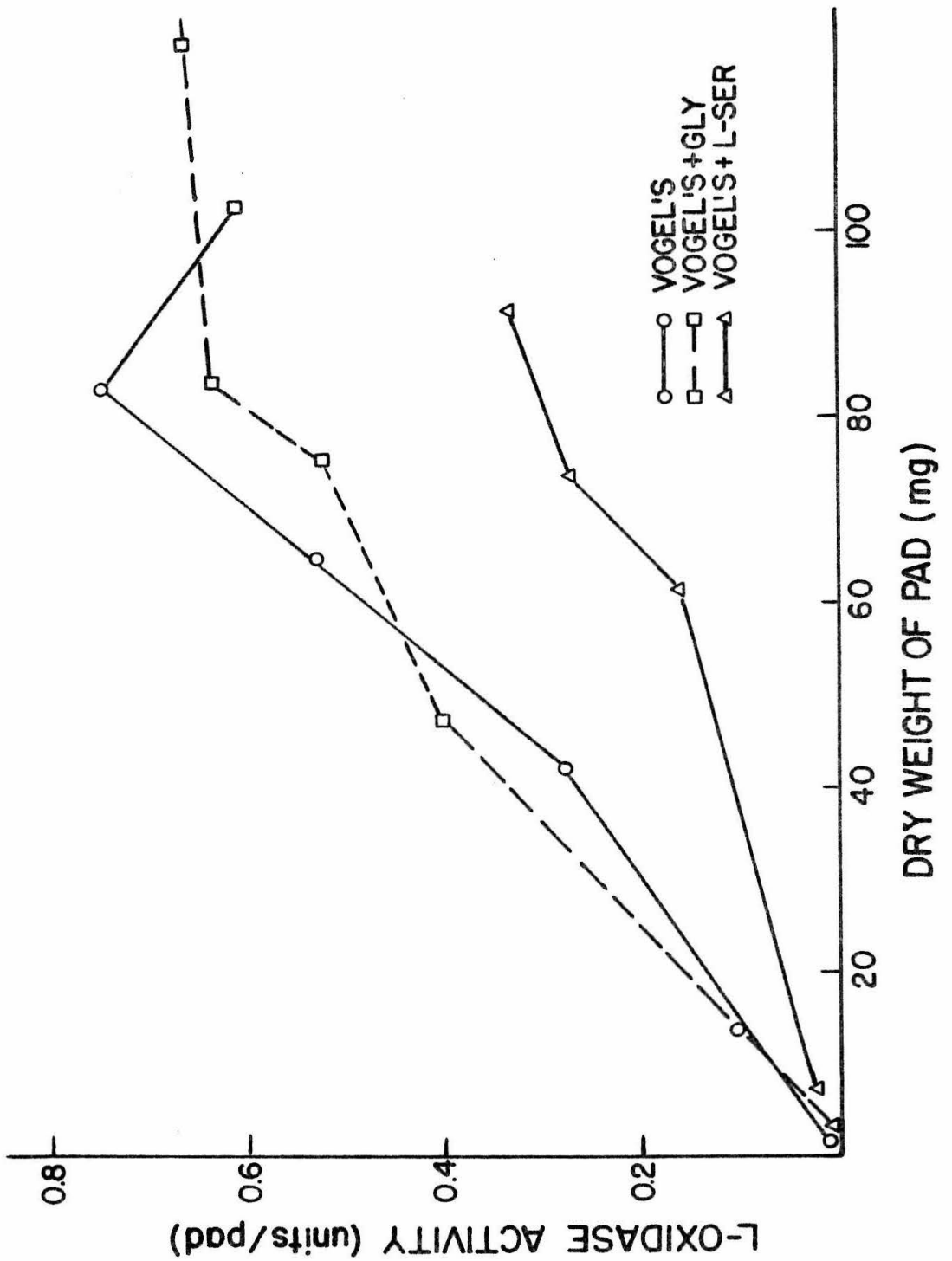

L-OXIDASE ACTIVITY (units/pad)
0.8
0.6
0.4
0.2
20
40
60
80
100
DRY WEIGHT OF PAD (mg)
VOGEL'S
VOGEL'S+GLY
VOGEL'S+ L-SER

TABLE XIV

The effect of serine and glycine on the L-oxidase reaction

Additions to the reaction mixture	L-oxidase (ECU/gm)	% Inhibition
none	4.3	
0.5 mg L-serine	3.9	9.3
1.0 mg L-serine	3.6	16
0.5 mg glycine	4.3	0
1.0 mg glycine	3.7	14

The results reported here confirm that P110 is a mutant incompletely blocked in the synthesis of serine and/or glycine. Production of the L-oxidase by P110 is reduced, but is not lowered to wild-type level, by supplying the mutant with serine.

Synthesis of L-Amino Acid Oxidase by Amino Acid Auxotrophs Other than P110

The finding that serine reduced the rate of oxidase synthesis by P110 suggested that the requirement for serine was directly related to synthesis of the oxidase. Each of the serine-requiring strains of Neurospora listed by the Fungal Genetics Stock Center was obtained and tested for synthesis of oxidase when grown on a limiting amount of serine. The results shown in Table XV indicate that each of the strains which responded to serine synthesized oxidase. Ser-2 did not respond to serine in my hands. The high level of L-oxidase produced by H605 ser-1 and C127 ser-1 grown on Vogel's minimal medium is reduced by the addition of serine, whereas 47903 ser-3 produced approximately the same amount of oxidase with or without serine present in the medium.

Whether synthesis of L-oxidase was restricted to serine auxotrophs was investigated on a number of other mutant strains grown in the presence of little or no supplement. The results, given in Table XVI, indicate that some mutant strains, but not all, synthesize the oxidase when growth is restricted by limiting supplement. Strains 38706 me-1, C167 tyr, H 98 me, 16117 ileu, his 1B-2A, and 35203 ad-3 did not produce more than a small amount of L-oxidase on any level of

TABLE XV

Synthesis of L-oxidase by serine-requiring mutants

Culture	Medium	Days growth	Dry wt, mg	L-oxidase ECU/gm
ser-1 (H605)	Vogel's	5	10.6	17.9
ser-1 (H605)	+ ser	5	70.0	3.1
ser-1 (C127)	Vogel's	5	8.0	14.8
ser-1 (C127)	+ ser	5	57.8	3.2
ser-2	Vogel's	3	79.2	0.09
ser-2	+ ser	3	64.0	0.18
ser-3	Vogel's	7	1.6	4.8
ser-3	+ ser	7	72.4	5.3

Cultures were grown without shaking in 20 ml medium in 125 ml Erlenmeyer flasks at 25°C. L-serine was added to give a final concentration of 0.2 mg/ml.

TABLE XVI

Synthesis of L-oxidase by auxotrophs other than P110

Culture	Supplement	Days growth	Wet wt, mg	L-oxidase, ECU/gm
38706 me	none	3	38	0.39
38706 me	low met	3	100	0.65
38706 me	high met	3	128	0.53
37501 leu	low leu	3	85	8.95
37501 leu	high leu	3	138	0.27
37811 lys	low lys	3	20	13.8
37811 lys	high lys	3	125	0.27
arg-1	none	7	80	11.1
arg-1	low arg	4	23	33.4
arg-1	high arg	4	175	2.8
arg-8	none	7	125	1.6
arg-8	low arg	4	80	4.2
arg-8	high arg	4	130	1.0
C167 tyr	none	7	38	0.91
C167 tyr	low tyr	4	135	0.11
C167 tyr	high tyr	4	150	0.08
H98 me	low met	4	148	1.4
H98 me	high met	4	188	0.94
16117 ileu	low ileu + val	4	58	1.2
16117 ileu	high ileu + val	4	178	0.13
his 1B-2A	low his	4	125	0.75
his 1B-2A	high his	4	108	0.96
35203 ad-3	low adenine	4	92	0.17
35203 ad-3	high adenine	4	192	0.09

Supplements were added to give a final concentration of 0.02 mg/ml (low level of supplement) or 0.2 mg/ml (high level of supplement). All cultures were grown at 25°C on 20 ml Vogel's medium in 125 ml Erlenmeyer flasks.

supplement investigated. Strains 37501 leu-2, 37811 lys, arg-1A and arg-8A are similar to the serine-requiring mutants in their synthesis of L-oxidase when grown on limiting amounts of their requirement. Thus the synthesis of L-oxidase is not restricted to serine-requiring strains, nor is it characteristic of all amino acid auxotrophs grown on suboptimal levels of supplement.

Strain 38706 me-1 was tested for its ability to synthesize L-oxidase when grown on aminobutyric acid as the sole nitrogen source, to assure that this strain was capable of synthesizing the enzyme under conditions which induce the wild-type. The mutant grew very slowly in the absence of methionine, but produced a high level of L-oxidase under these conditions. When methionine (0.02 mg/ml) and aminobutyric acid (0.5 mg/ml) were both added to the nitrogen-free medium, the culture grew 42 mg wet weight in 4 days and produced 15.7 ECU/gm L-oxidase. Thus, the lack of oxidase in 38706 grown on limiting supplement is not due to the inability of the strain to produce L-oxidase under any condition.

A Comparison of L-Oxidase in Shaken Versus Standing Cultures

Burton observed that induction of L-oxidase in cultures grown on low biotin and casamino acids was prevented by shaking the cultures (3). Derepression of a high level of oxidase occurs in shaken cultures in the method described by Pall (41). It was of interest to determine whether production of L-oxidase by P110 was prevented by shaking the cultures, similar to the case in induced wild-type, or was enhanced

by shaking, as in the derepressed cultures. As shown in Table XVII, shaking P110 or II6 greatly reduces the amount of oxidase produced by the mutants. Thus, with respect to the effects of shaking on production of the L-oxidase, the constitutive mutant behaves like an induced culture.

The response to serine and glycine is not eliminated by shaking the culture, as shown in Table XVIII, although growth is significantly improved by shaking.

Other auxotrophic strains were tested for the effects of shaking on their growth and formation of L-oxidase. The results of this study are shown in Table XIX. Both H 605 ser-1 and C 127 ser-1 showed an improvement in growth and produced less L-oxidase in shaken cultures than in standing cultures. The mutant ser-3 grew very little in standing cultures, and shaking cultures grew only after a long lag period. Less L-oxidase is found in shaken cultures of ser-3 than in standing cultures, but the amount of L-oxidase found in the shaken cultures is significantly greater than the basal levels of L-oxidase produced by wild-type strains. These results indicate that the degree to which oxidase production is reduced by shaking varies from strain to strain, even within the strains requiring serine. Shaken cultures of arg-1 grew significantly better than stationary cultures and contained little L-oxidase. As a rule, a rapid rate of growth is associated with a decrease in the production of L-oxidase.

TABLE XVII

The effects of shaking on growth and L-oxidase production by P110 and II6a;cot

Culture	Growth in days	Wet wt (mg)	Oxidase activity (ECU/gm)
P110	3 days standing	20	2.45
P110	3 days shaken	75	0.27
P110	5 days standing	60	2.58
II6	5 1/2 days standing	40	10.3
II6	5 1/2 days shaken	190	0.51

Cultures were grown in 20 ml Vogel's minimal medium in 125 ml Erlenmeyer flasks at 25°C, stationary or on a reciprocal shaker.

TABLE XVIII

The effect of shaking on the serine or glycine requirement of P110

Additions to Vogel's medium	Conditions	P110	ST 74A
		Dry wt (mg)	Dry wt (mg)
none	Standing	13.7	112.0
5 μmoles/ml L-serine	Standing	60.8	107.4
5 μmoles/ml glycine	Standing	46.9	93.9
none	Shaken	74.3	179.2
5 μmoles/ml L-serine	Shaken	140.1	182.7
5 μmoles/ml glycine	Shaken	153.3	172.4

Cultures were grown for 4 days in 20 ml medium in 125 ml Erlenmeyer flasks at 25°C. All values given in the table are averages of duplicate cultures.

TABLE XIX

The effects of shaking on growth and L-oxidase production by various auxotrophs grown without supplement

Culture	Growth in days	Dry wt (mg)	Oxidase activity (ECU/gm)
ST 74A	4 days standing	91.1	0.054
ST 74A	4 days shaking	143.3	0.040
P110	4 days standing	18.9	7.55
P110	4 days shaking	87.1	0.15
H 605	4 days standing	10.1	17.5
H 605	4 days shaking	75.3	0.36
C 127	4 days standing	5.9	5.9
C 127	4 days shaking	39.8	0.06
ser-3	7 days standing	2.6	6.51
ser-3	7 days shaking	26.8	2.65
arg-1	7 days standing	11.9	13.1
arg-1	7 days shaking	114.2	0.29

Cultures were grown in 20 ml Vogel's medium in 125 ml Erlenmeyer flasks at 25°C, stationary or on a reciprocal shaker. All values are averages of duplicate pads.

A Comparison of Serine Biosynthetic Enzymes in P110 and in Wild-type Cultures

The pathways for synthesis of serine in Neurospora crassa have been studied by Sojka and Garner (25). According to these authors, serine is synthesized from two products of glycolysis, phosphoglyceric acid and glyceric acid (Figure 1). The phosphorylated pathway appears to predominate in cultures grown on either glucose or fructose, but the non-phosphorylated pathway is enhanced by growth on fructose.

Each enzyme in the two pathways described by Sojka and Garner was prepared from P110 and compared to the same enzyme prepared from ST 74A. A summary of the results obtained for glyceric acid dehydrogenase (GLAD) is given in Table XX. All enzyme activities are expressed as μmoles NADH oxidized per mg protein per hr. On Westergaard-Mitchell medium supplemented with fructose, less GLAD activity was observed in P110 than in ST 74A. As observed by Sojka and Garner (25), GLAD activity was reduced in wild-type grown on glucose, but glucose did not reduce the level of GLAD seen in P110, so that the GLAD activities in the two cultures grown on glucose are very similar. Likewise, the GLAD activities of the two cultures grown in Vogel's medium containing sucrose do not differ significantly.

In Table XXI, phosphoglyceric acid dehydrogenase (PGLAD) activities in ST 74A and P110 are compared. When grown on fructose, P110 shows approximately twice the PGLAD activity observed in ST 74A. The difference between the two cultures is less marked when they are

TABLE XX

A comparison of glyceric acid dehydrogenase activities in P110 and ST 74A

Culture	Carbon source	Hours growth	Test	Control	Specific activity
			μmoles/mg protein/hr	μmoles/mg protein/hr	μmoles/mg protein/hr
ST 74A	fructose	19	0.91	0.431	0.479
	fructose	19	0.696	0.348	0.348
	fructose	18	0.965	0.496	0.469
P110	fructose*	19	0.356	0.220	0.132
	fructose	65	0.85	0.76	0.09
	fructose	43	0.178	0.119	0.059
	fructose	42	0.382	0.306	0.076
ST 74A	glucose	19	0.423	0.222	0.218
	glucose	18	0.581	0.443	0.138
P110	glucose	43	0.396	0.226	0.170
	glucose	42	0.457	0.403	0.054
ST 74A	sucrose**	44	0.354	0.152	0.202
	sucrose**	42	0.228	0.142	0.086
P110	sucrose**	145	0.312	0.250	0.062
	sucrose**	90	0.193	0.121	0.072

*Medium contained 0.1 mg/ml L-serine.

**Vogel's medium replaced Westergaard-Mitchell medium. Cultures were grown in 20 ml medium in 125 ml Erlenmeyer flasks, without shaking at 25°C. Cultures were grown in 750 ml Westergaard-Mitchell medium in 2 liter low form culture flasks with shaking at 25°C.

TABLE XXI

A comparison of phosphoglyceric acid dehydrogenase activity in P110 and ST 74A

Culture	Carbon source	Hours growth	Test	Control	Specific activity
			μmoles/mg protein/hr	μmoles/mg protein/hr	μmoles/mg protein/hr
ST 74A	fructose	19	1.15	0.259	0.891
	fructose	19	1.22	0.348	0.874
	fructose	19	1.314	0.496	0.818
P110	fructose	65	2.46	0.471	1.989
	fructose	43	2.02	0.119	1.901
	fructose	42	2.010	0.306	1.704
ST 74A	glucose	19	1.25	0.222	1.028
	glucose	18	1.162	0.443	0.719
P110	glucose	43	2.48	0.226	2.254
	glucose	42	1.720	0.403	1.317
ST 74A	sucrose*	44	1.265	0.152	1.113
	sucrose*	42	1.225	0.142	1.083
P110	sucrose*	115	2.22	0.250	1.97
	sucrose	90	1.305	0.121	1.184

*Vogel's medium replaced Westergaard-Mitchell medium. Cultures were grown in 20 ml medium in 125 ml Erlenmeyer flasks, without shaking at 25°C. Cultures were grown in 750 ml Westergaard-Mitchell medium in 2 liter low form culture flasks with shaking at 25°C.

grown on glucose or sucrose, but the activity in P110 is consistently higher than the activity of PGLAD in ST 74A.

The next enzymes compared were the serine transaminases and phosphoserine transaminases. As shown in Figure 14, no serine transaminase activity was demonstrable in ST 74A within the 15 minute reaction period. The reactions with P110 extracts were monitored for 30 minutes, by which time both serine and phosphoserine transaminase activities were apparent (Figure 15). The specific activity of phosphoserine transaminase in P110 was 7.87×10^{-3} μmoles glutamate/mg protein/hr compared to 7.91×10^{-3} μmoles/mg/hr in ST 74A. Serine transaminase activity in P110 was 1.14×10^{-3} μmoles/mg/hr. Clearly, neither of these enzymes is deficient in P110.

The last enzyme investigated was phosphoserine phosphatase. The activity in both strains was higher than that reported by Sojka and Garner (25) for wild-type Li 1A (1.35 μmoles/mg/hr). P110's activity was 5.92 μmoles/mg/hr compared to an activity of 3.43 μmoles/mg/hr in ST 74A.

In summary, none of the enzymes involved in the synthesis of serine from the products of glycolysis is deficient in P110. Only glyceric acid dehydrogenase showed a lower activity in P110 than in ST 74A, and this difference was decreased by growing the cultures on glucose minimal medium. The low level of GLAD in P110 grown on fructose probably does not account for the serine requirement of the mutant; wild-type cultures having a comparable level of GLAD activity on glucose medium show no serine requirement.

Figure 14. Phosphoserine transaminase and serine transaminase activities in ST 74A. Enzyme activity is measured by the formation of C^{14}-glutamic acid by transamination of labeled α-ketoglutaric acid in the presence of enzyme and phosphoserine or serine. The radioactive glutamic acid product was isolated on a small Dowex column and counted. Each point on the graph represents the amount of glutamic acid produced in a reaction mixture containing approximately 5 mg protein from a crude mycelial extract.

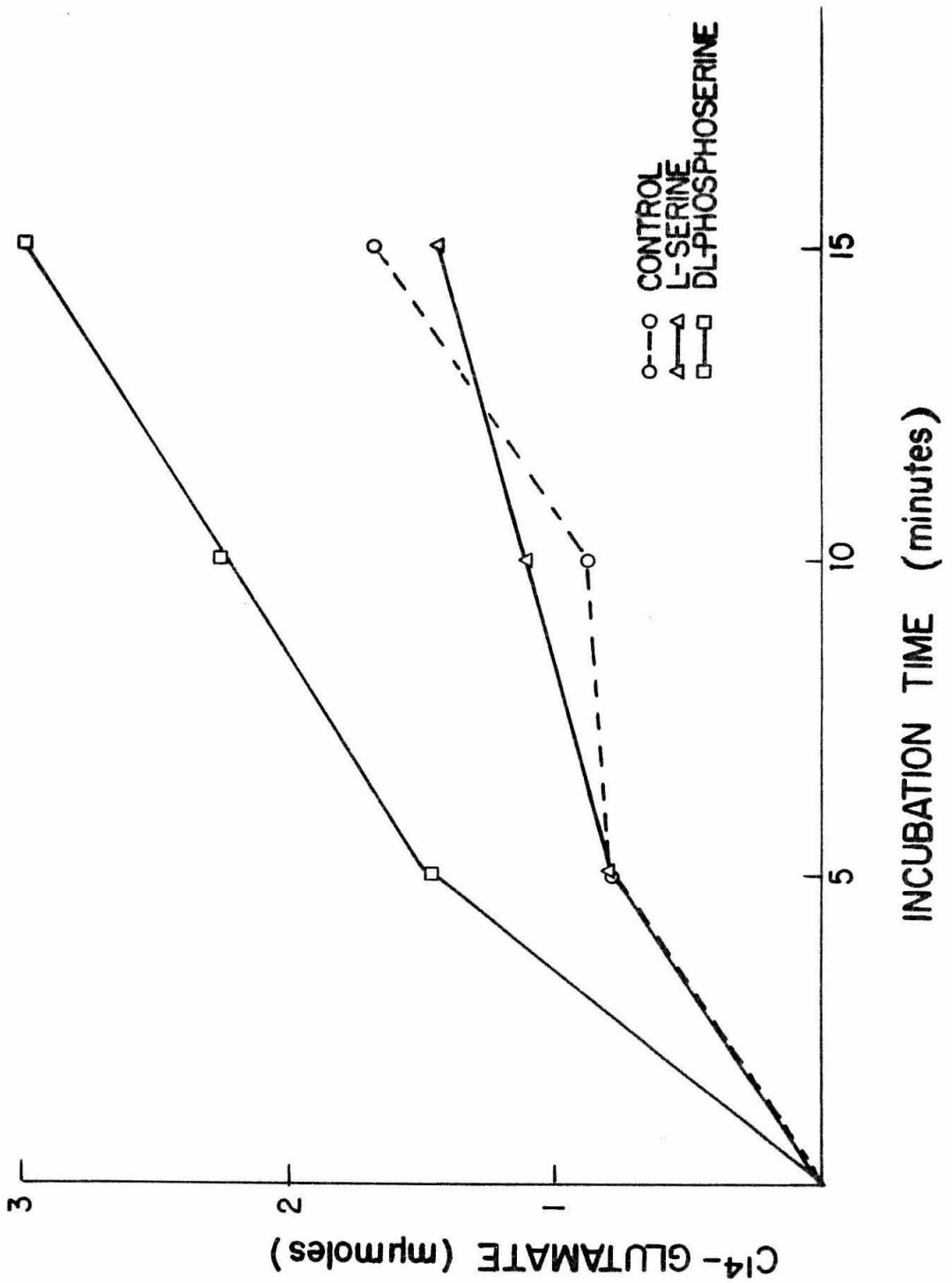
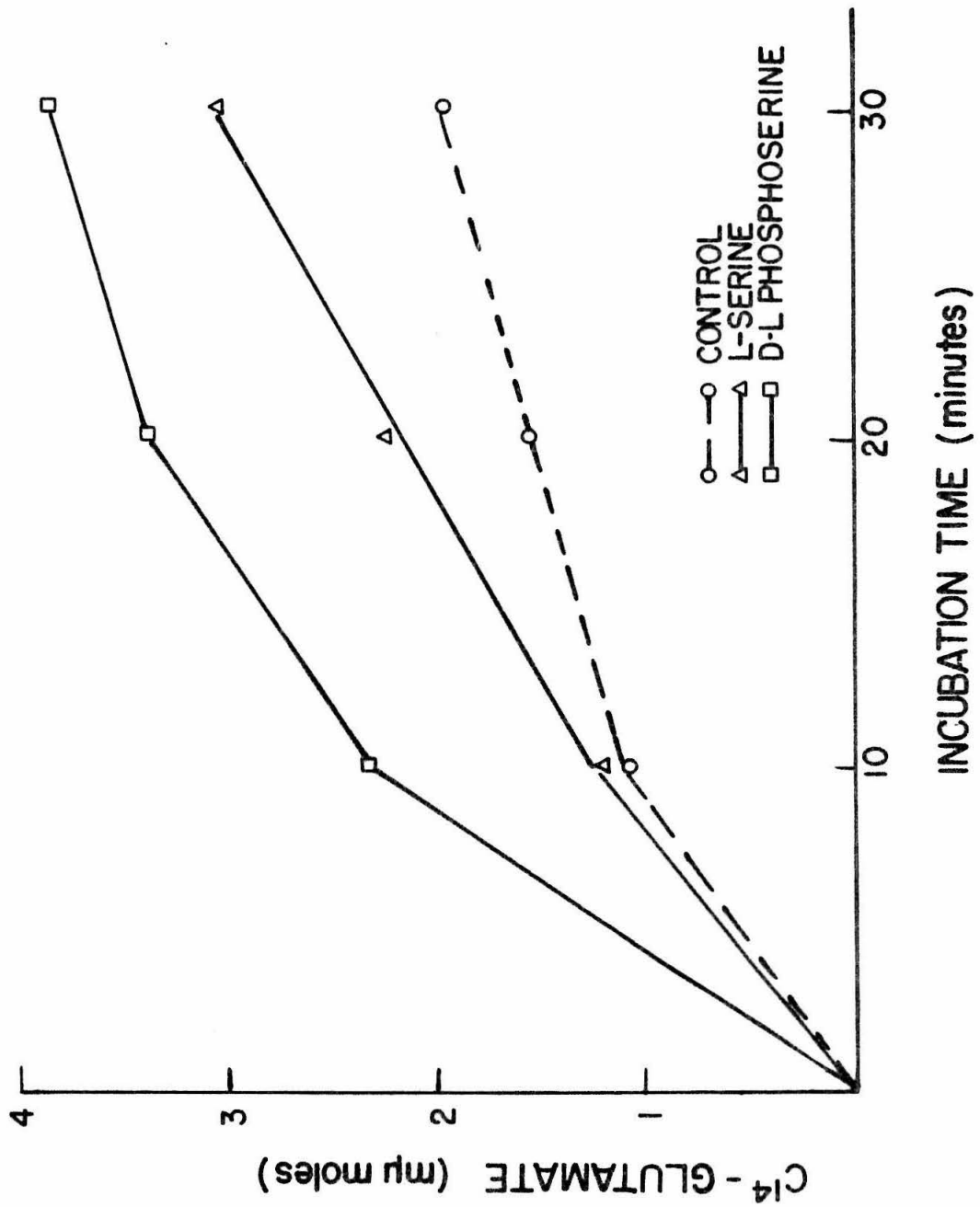

C14- GLUTAMATE (mμmoles)
3
2
1
5
10
15
INCUBATION TIME (minutes)
CONTROL
L-SERINE
DL-PHOSPHOSERINE

Figure 15. Phosphoserine transaminase and serine transaminase activities in P110. Enzyme activity is measured by the formation of C^{14}-glutamic acid by transamination of labeled α-ketoglutaric acid in the presence of enzyme and phosphoserine or serine. The radioactive glutamic acid product was isolated on a small Dowex column and counted. Each point on the graph represents the amount of glutamic acid produced in a reaction mixture containing approximately 5 mg protein from a crude mycelial extract.


C^{14} - GLUTAMATE (mμ moles)
4
3
2
1
10
20
30
INCUBATION TIME (minutes)
CONTROL
L-SERINE
D-L PHOSPHOSERINE

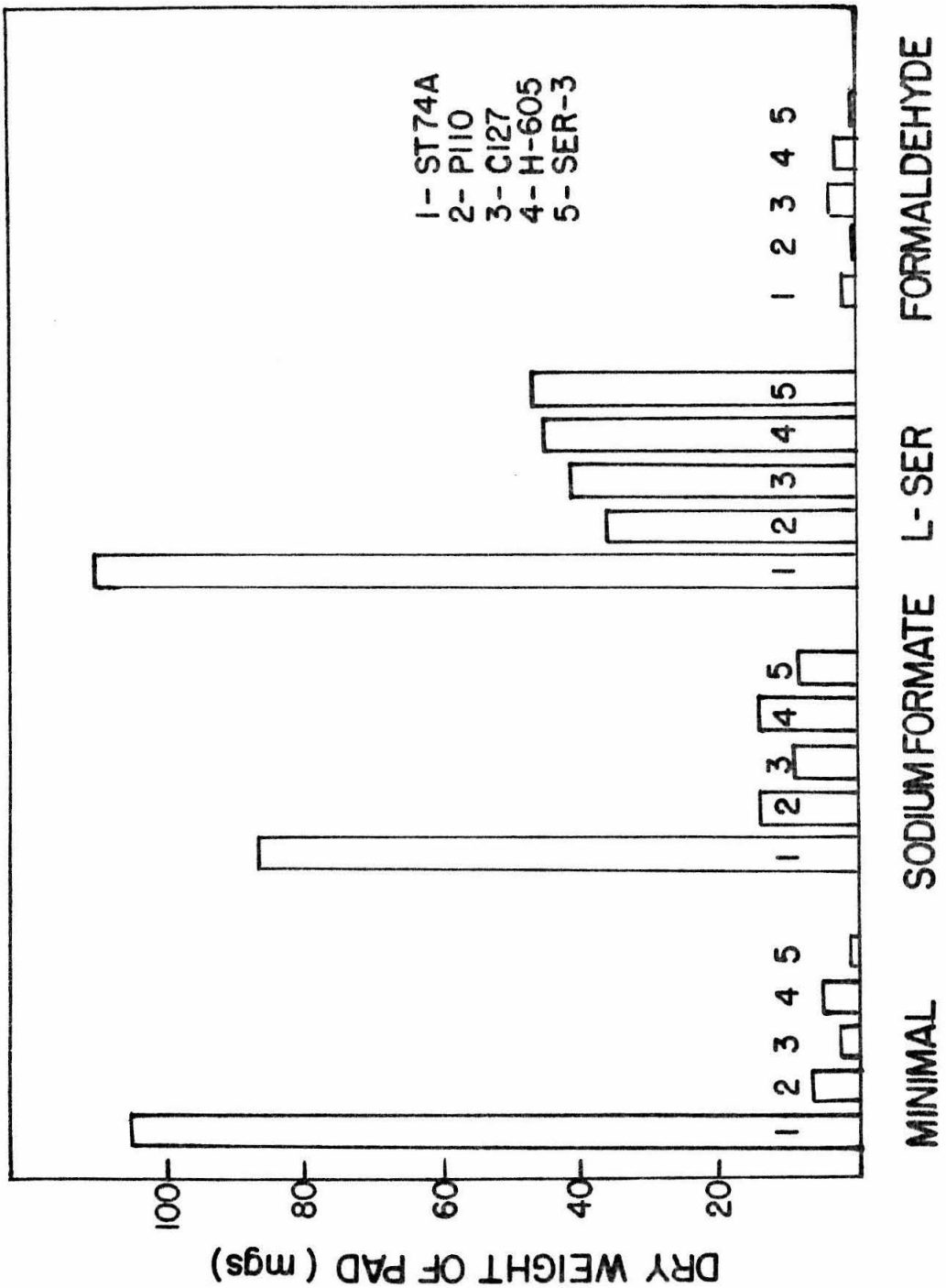
Growth Studies Comparing P110 and Other Serineless Strains

The comparison of serine biosynthetic enzyme activities in P110 and ST 74A suggested that P110 might not require serine itself, but some compound derived from serine or a compound which is spared by serine. The results of growth studies reported earlier in this thesis suggested that P110 might be deficient in single-carbon (C_1) units, utilized in the synthesis of methionine, thymine, and purines. To test this possibility, growth of P110 was determined on media containing sodium formate, formaldehyde, or L-serine at concentrations varying from 1.25 to 2.5 μmoles/ml. All cultures were grown at pH 4.5 to facilitate the uptake of formate (69). For comparison, cultures of ST 74A and each of the known serineless mutants were grown on identical media. Formaldehyde was inhibitory to all cultures at all concentrations investigated. The two isolates of ser-1 grew better on formaldehyde than did ST 74A, but none of the cultures grew more than 5 mg in 4 days. The results shown in Figure 16 indicate that formate is less efficient, mole for mole, than serine in supporting the growth of P110 or any other serineless mutant.

The active C_1 unit in metabolism is a folic acid derivative (71-75). If P110 were deficient in folic acid, feeding formate would have no effect on growth. Folic acid added to minimal medium at concentrations ranging from 4×10^{-5} μg/ml to 4 μg/ml did not enhance growth of P110.

All studies carried out by Sojka and Garner (25) had utilized in vitro assays for identification of the biochemical pathways leading

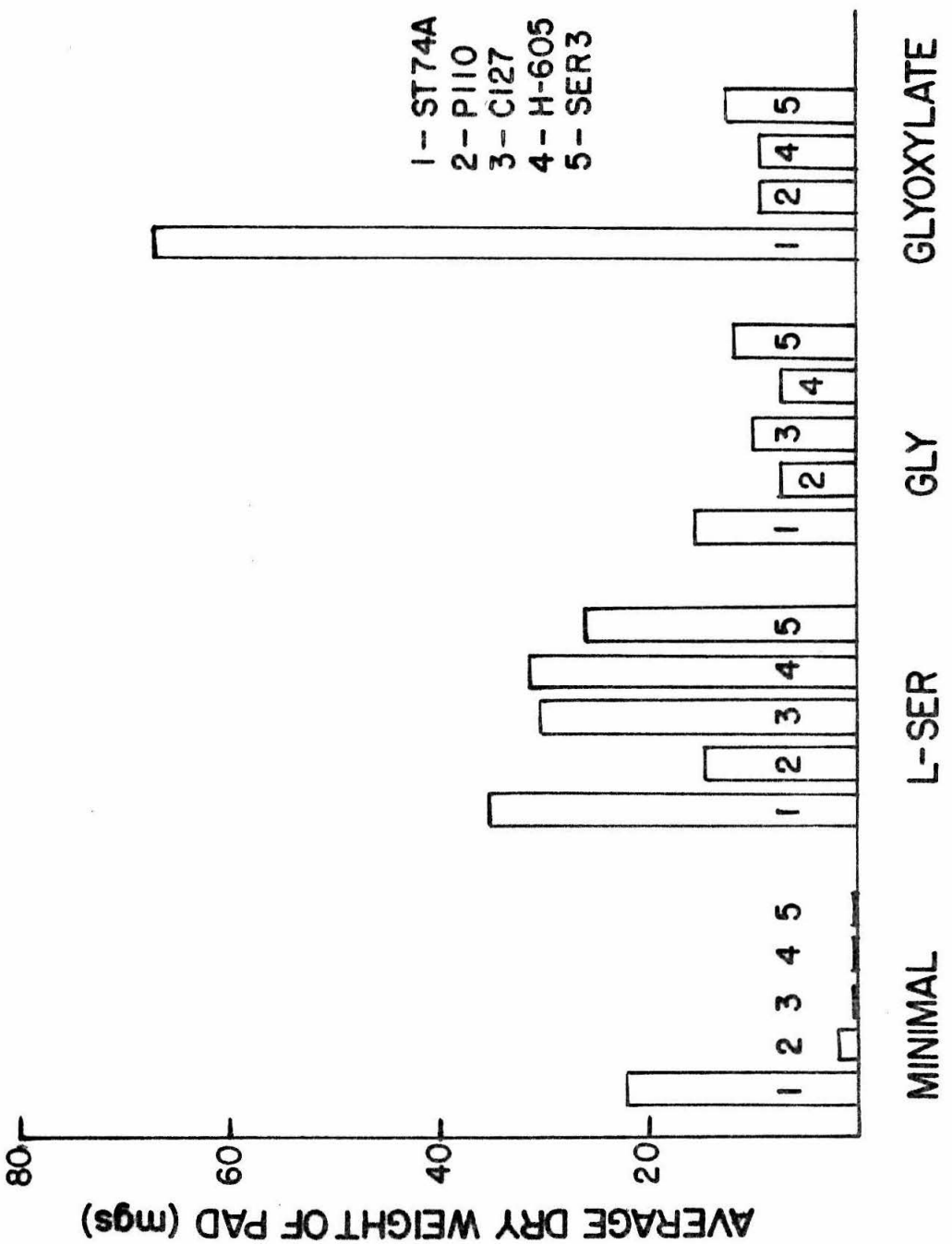
Figure 16. Growth of serine-requiring mutants on sodium formate, formaldehyde, and serine. All cultures were grown on 20 ml Vogel's minimal medium in 125 ml Erlenmeyer flasks. The pH of the medium was adjusted to 4.5 with concentrated HCl to facilitate the uptake of formate. Formaldehyde was added to give a final concentration of 1.25 μmoles/ml. Sodium formate or serine was added to give a final concentration of 2.5 μmoles/ml. All cultures were incubated without shaking at 25°C for 4 days.


DRY WEIGHT OF PAD (mgs)
100
80
60
40
20
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
1- ST74A
2- P110
3- C127
4- H-605
5- SER-3
MINIMAL
SODIUM FORMATE
L-SER
FORMALDEHYDE

to serine in Neurospora. The observation that serine is synthesized from glycine in baker's yeast (33) suggested that the pathways leading to serine from phosphoglyceric acid and glyceric acid might play minor roles in vivo in Neurospora, and that glycine was the major source of serine. Labeling studies reported in a previous section of this thesis indicated that P110 could convert glycine to serine readily. Wright (34) had reported that a serine- or glycine-requiring mutant investigated by her could utilize glyoxylic acid (HCOCOOH) or glycolate ($HOCH_2COOH$) as efficiently as glycine or serine, suggesting that serine might be synthesized from products of the tricarboxylic acid cycle. Preliminary studies indicated that none of the serine-requiring mutants investigated here could utilize glyoxylate or glycolate for growth. If, however, the carbon source was changed from glucose to glycerol (Wright used a glycerol minimal medium), glyoxylate was utilized as well as glycine by all of the serineless strains. A comparison of the growth of each strain on minimal, serine, glycine, and glyoxylate is given in Figure 17. P110 grew less on serine than did the other strains, but all strains grew equally poorly on glycine. Apparently, ST 74A utilized glyoxylate as a carbon source in the presence of glycerol, whereas there was no evidence that the mutants did so. Growth of the mutants was probably limited by the supply of glycine produced from glyoxylate. Note the equivalence in growth on glycine compared to glyoxylate for each mutant strain.

If P110 were blocked at some step in glycolysis prior to the formation of glyceric acid, compounds in addition to serine should be

Figure 17. Growth of serine-requiring mutants on minimal medium compared to growth on medium supplemented with L-serine, glycine or glyoxylate. Cultures were grown on 20 ml Vogel's medium and 1 per cent glycerol in 125 ml Erlenmeyer flasks. The pH of the medium was adjusted to 4.5 with concentrated HCl. Each of the supplements was added to give a final concentration of 5 μmoles/ml. All cultures were incubated at 25°C without shaking for 6 days. Dry weights plotted are averages of two pads in each case.


AVERAGE DRY WEIGHT OF PAD (mgs)
80
60
40
20
1 2 3 4 5
MINIMAL
L-SER
GLY
GLYOXYLATE
1 - ST74A
2 - P110
3 - C127
4 - H-605
5 - SER3

required for growth. Pyruvate would be expected to be the major metabolic intermediate lacking in such a mutant. Pyruvate has been reported to improve the growth of P110 grown on minimal medium containing sucrose (7), although the increase in dry weight was not reported. In the experiment shown in Figure 18, none of the serine-requiring strains studied grew more than 5 mg dry weight in 6 days on pyruvate and glycerol, although ST 74A grew more than 85 mg on this medium. Adding pyruvate to serine-containing medium had no effect on the mutants, as expected if their growth is limited by serine. ST 74A has grown better on serine plus pyruvate than on serine alone, indicating that pyruvate is used as a carbon source by ST 74A under these conditions. Adding serine to pyruvate-containing medium appears to inhibit utilization of pyruvate by ST 74A. There is no evidence that any of the serine-requiring mutants is blocked at a step in glycolysis prior to formation of glyceric acid.

Enhancement of the growth of P110 by glycine and methionine was described previously in this thesis. To see whether P110 differed from the other serineless mutants in this property, each of the strains was grown on the media indicated in Figure 19. P110 showed the greatest enhancement by glycine and methionine of any of the strains investigated. In this experiment, P110 grew no better on glycine plus serine than on serine alone, probably because serine was supplied at its optimum concentration. In earlier experiments, serine was added at suboptimal concentrations. The difference between P110 and the other strains is particularly notable on serine and methionine. Adding methionine to

Figure 18. Growth of serine-requiring mutants on pyruvate in the presence and absence of serine. Cultures were grown for 6 days at 25°C on 20 ml Vogel's medium and 1 per cent glycerol in 125 ml Erlenmeyer flasks. The pH of the medium was adjusted to 4.5 with concentrated HCl, prior to autoclaving the flasks. Serine was added to give a final concentration of 5 μmoles/ml. Twenty mg filter-sterilized sodium pyruvate was added each day for 4 days to each flask supplemented with pyruvate. Dry weights given are averages for duplicate pads in each case.

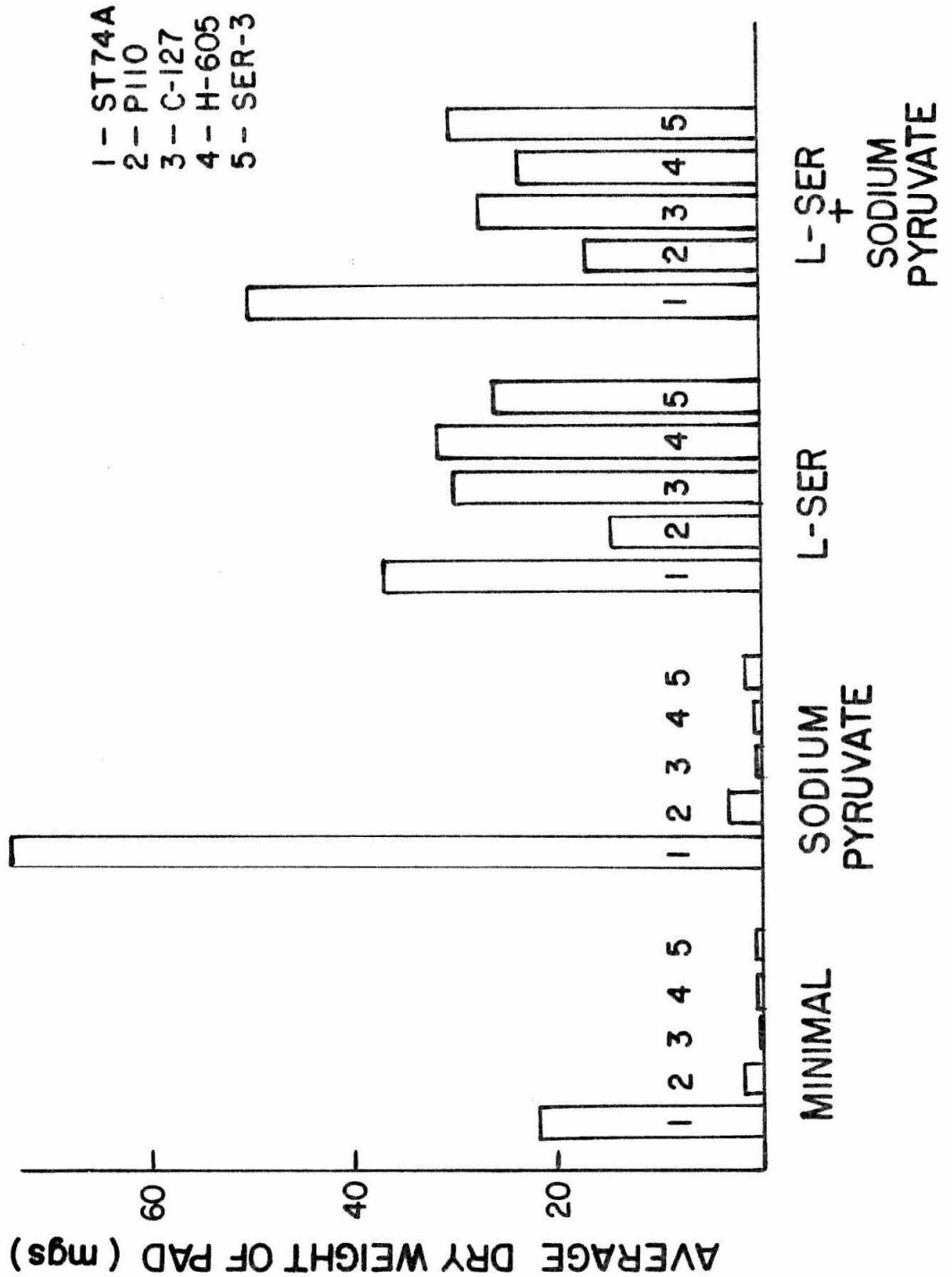
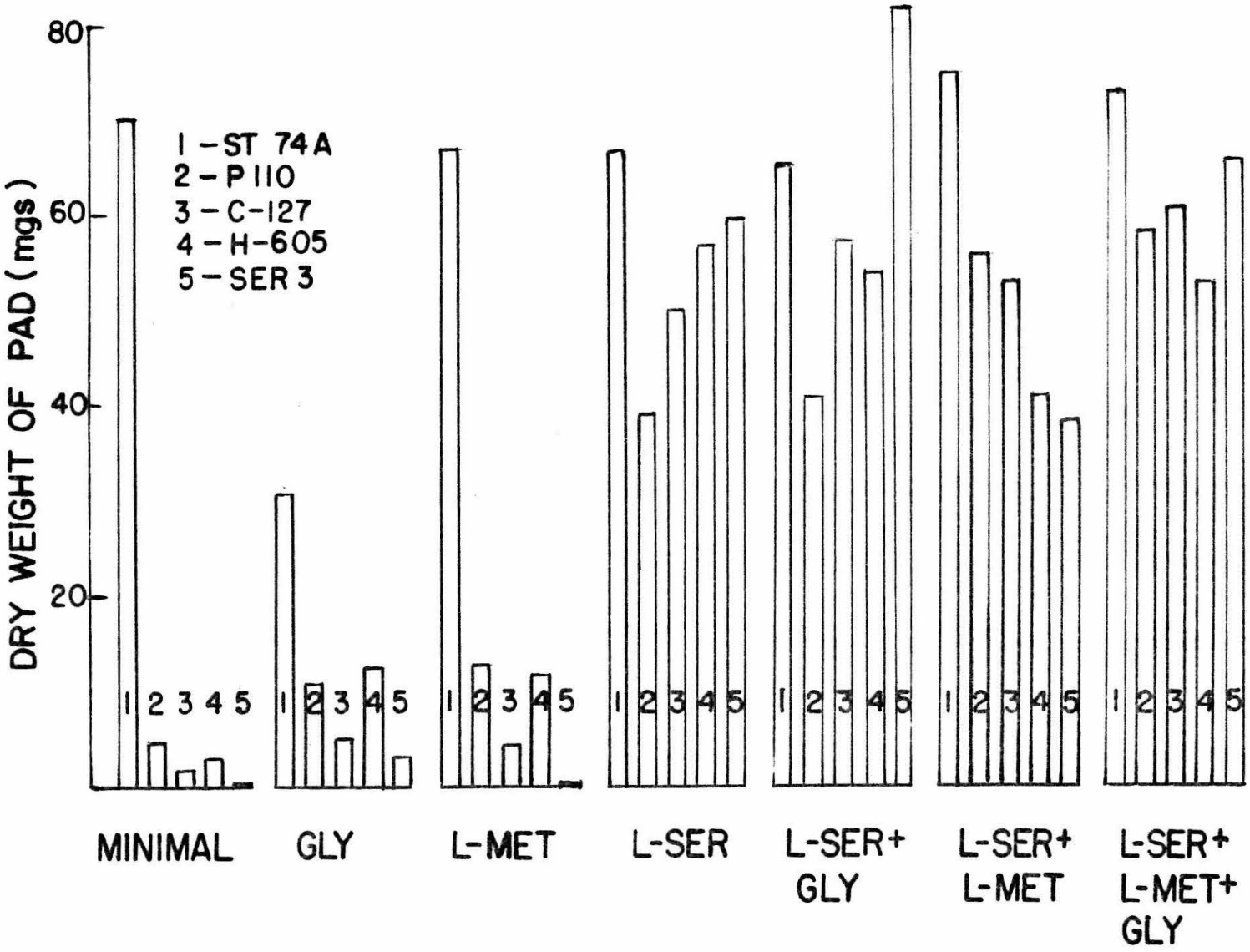

1 – ST74A
2 – P110
3 – C-127
4 – H-605
5 – SER-3
AVERAGE DRY WEIGHT OF PAD (mgs)
60
40
20
MINIMAL
SODIUM PYRUVATE
L-SER
L-SER + SODIUM PYRUVATE

Figure 19. Growth of serine-requiring mutants on serine and metabolites related to serine. All cultures were grown for 3 days at 25°C on Vogel's medium and 2 per cent sucrose in 125 ml Erlenmeyer flasks. Each supplement was added to give a final concentration of 5 μmoles/ml.


DRY WEIGHT OF PAD (mgs)
80
60
40
20
1 - ST 74A
2 - P110
3 - C-127
4 - H-605
5 - SER 3
1 2 3 4 5
MINIMAL
GLY
L-MET
L-SER
L-SER+ GLY
L-SER+ L-MET
L-SER+ L-MET+ GLY

serine caused a significant increase in the dry weight of P110, whereas H 605 ser-1 and 47903 ser-3 grew less well with added methionine than on serine alone. Adding glycine and methionine to serine-supplemented medium allowed each of the mutants to grow nearly as well as wild-type.

None of the growth tests performed indicated differences among the serine-requiring strains, with the exception of the effect of methionine on P110. This observation may indicate that P110 is deficient in some aspect of C_1 metabolism which can be compensated by supplying serine and methionine, but which is not compensated by formate. An alternative explanation for the enhancement of growth of P110 by methionine is suggested by results of an analysis of the amino acid pools of P110.

A Comparison of the Amino Acid Pools of ST 74A and P110

Sojka reported that each of the serine-requiring mutants studied by him contained significantly less than the wild-type concentrations of free intracellular glycine and serine (77). Sojka backcrossed H 605 ser-1, C 127 ser-1, ser-2, and ser-3 to wild-type Li-1 three times to minimize differences in their pools due to possible differences in the parental strains from which these mutants were derived. The glycine concentrations in reisolates of the serine auxotrophs grown to 60 mg dry weight in standing cultures ranged from 33 to 48 per cent of the concentration in wild-type Li-1 (77). Serine ranged from 58 to 63 per cent of the wild-type concentration. Amino acids were analyzed on an

amino acid analyzer. The concentrations of other amino acids were not reported.

To determine whether P110 was similar to other serine-requiring strains with respect to changes in its glycine and serine pools, the free intracellular amino acid pools were extracted from pads of ST 74A and P110 grown without shaking at 25°C. Individual amino acids were separated by high voltage electrophoresis. After staining the electrophorogram with cadmium-ninhydrin to visualize the locations of each amino acid, the spots were cut out and eluted with methanol. The concentration of each amino acid was estimated from the intensity of the color eluted from the spot. The results of this study are shown in Table XXII. The glycine concentration in P110 is about 46 per cent of that found in wild-type ST 74A. Thus P110 is similar to other serine-requiring strains in its deficient free glycine pool. A comparison of the serine concentration in P110 and ST 74A is confused by the accumulation of compounds in P110 which migrate to the same region as serine during high voltage electrophoresis. These compounds will be discussed in detail below.

Two very striking changes in the free amino acid pools are observed in P110 in addition to the low glycine pool. The alanine pool in P110 is only 13 per cent of the alanine pool in ST 74A. Umbarger has reported that alanine production from pyruvate does not appear to be regulated except by the extent to which pyruvate is accumulating as a catabolic intermediate, the amount of nitrogen available, and the extent to which reducing power (NADPH) is available for reductive

TABLE XXII

A comparison of the amino acid pools in P110 and ST 74A grown without shaking

Amino acid	P110 Set I µmoles/gm	P110 Set II µmoles/gm	P110 Av. µmoles/gm	74A Set I µmoles/gm	74A Set II µmoles/gm	74A Av. µmoles/gm
Alanine	38.79	48.13	43.46	307.99	358.23	333.11
Arginine	29.64	33.48	31.56	33.29	37.20	35.24
Aspartic acid	8.01	8.67	8.34	4.88	5.40	5.14
Glutamic acid	16.30	14.46	15.38	28.96	30.52	29.74
Glutamine	52.08	55.11	53.60	58.81	95.97	77.39
Glycine	8.70	7.17	7.94	17.63	16.92	17.28
Histidine	10.28	9.67	9.98	9.59	7.29	8.44
Isoleucine	10.58	9.73	10.16	--	--	--
Valine	--	--	--	14.66	15.59	15.12
Leucine	5.21	3.58	4.40	2.30	2.54	2.42
Lysine	32.49	35.45	33.97	31.91	29.70	30.80
Methionine	10.20	11.03	10.62	9.53	10.06	9.80
Phenylalanine	--	--	--	6.47	6.78	6.62
"Serine" *	47.54	41.75	44.64	10.04	11.10	10.57
Threonine	46.08	45.18	45.63	5.70	5.10	5.40

TABLE XXII (continued)

Cultures were grown in 20 ml Vogel's minimal medium and 2 per cent sucrose in 125 ml Erlenmeyer flasks without shaking at 25°C until they had attained a dry weight of 30-40 mg (dry weight after amino acid extraction). Amino acids were extracted in 10 ml boiling distilled water for 30 minutes. Debris was removed by centrifugation and the supernatants were evaporated to dryness. Each sample was dissolved in 0.5 ml distilled water. Amino acids present in a 10 µl aliquot of the concentrated sample were separated by high voltage electrophoresis. Estimates of the concentrations of amino acids were made as described in the text.

*The figures given as "serine" concentrations include compounds other than serine which are accumulated by P110.

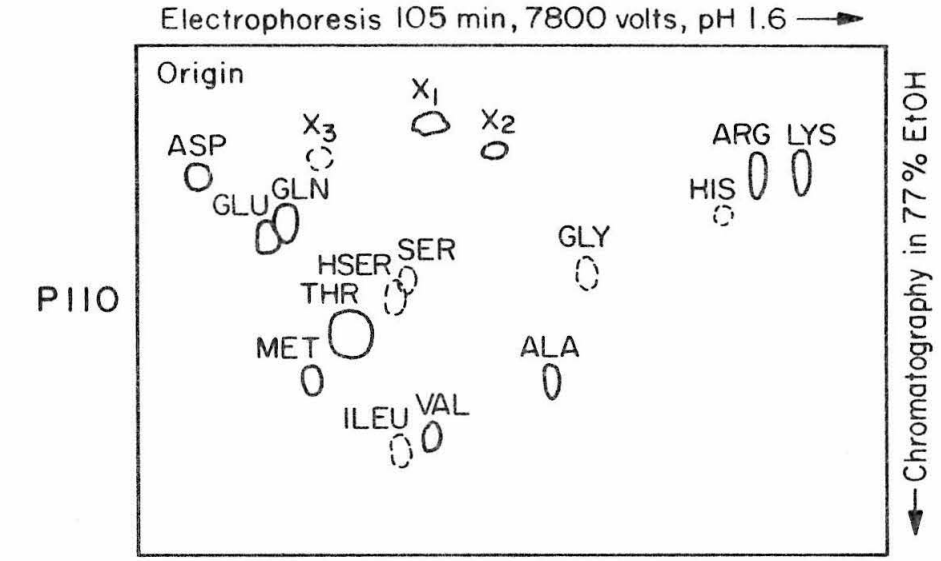
amination (103). The low alanine pool in P110 may result from a reduced supply of pyruvate arising from serine catabolism. Threonine is accumulated by P110 to yield a pool which is 8.5 times the concentration of threonine in ST 74A. Less striking changes are observed in aspartic acid and leucine, which are higher in P110 than in ST 74A, and in glutamic acid, which is lower in P110 than in ST 74A. Phenylalanine is missing in P110 and isoleucine is absent from ST 74A. Valine in P110 is indistinguishable from the "serine" spot. An unexpected finding was that the methionine pool of P110 is equivalent to that in ST 74A, despite the observed enhancement of growth of P110 on methionine in the presence of serine. Exogenous methionine may act to balance the levels of methionine and threonine in the cell. Other investigators (78) have reported antagonism between threonine and methionine in Neurospora.

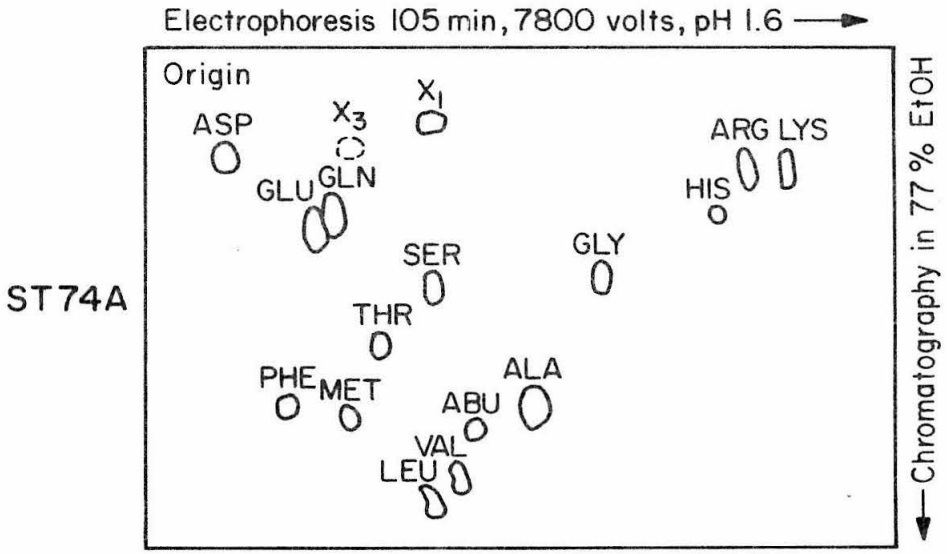
The results obtained using high voltage electrophoresis indicated that P110 accumulated serine in its free amino acid pool. This observation was inconsistent with the serine requirement of P110, and with the low glycine pool observed in the mutant. Evidence that the compounds which migrate (electrophoretically) like serine are not serine comes from a two-dimensional separation of the amino acids. The amino acids were first separated by high voltage electrophoresis. The unstained strip of separated amino acids was cut from the electrophorogram and sewn across the top of a second sheet of Whatman #3MM filter paper. The amino acids were then separated by descending chromatography in 77 per cent ethanol. The patterns of amino acid spots observed in P110 and ST 74A are shown diagrammatically in

Figure 20. ST 74A has a single discrete spot corresponding to serine, while P110 has two spots in the same region. One of these spots correlates with serine in ST 74A, and is notably less intense in P110 than in ST 74A. The second spot in this region has migrated faster than serine, and has the chromatographic properties of homoserine. Additional evidence that the compound is homoserine will be presented below. Another compound which migrated in the region of serine during electrophoresis has remained near the origin during chromatography. The compound (designated X_1 in the diagram) is present in both wild-type and P110, but appears to be accumulated by P110. This compound was not studied further. Other amino acids confirm the results obtained with high voltage electrophoresis. In particular, threonine is present in high concentration and alanine is present in low concentration in P110 compared to wild-type. P110 also differs from ST 74A in that aminobutyric acid is found in the wild-type, but not in P110. A compound which migrates electrophoretically like aminobutyric acid is found in P110, but chromatographically, this compound (designated X_2 in the diagram) is separable from aminobutyric acid. The absence of aminobutyric acid from P110 may result from its degradation by the L-amino acid oxidase present in P110. The nature of the compound labeled X_2 is unknown.

The results of high voltage electrophoresis and chromatography in 77 per cent ethanol indicated that P110 accumulated homoserine. The identification of homoserine was confirmed by additional tests. Homoserine is converted to homoserine lactone by acid hydrolysis (81).

Figure 20. A comparison of the amino acid pools of P110 and ST 74A. Cultures were grown for 3 days in 20 ml Vogel's minimal medium in 125 ml Erlenmeyer flasks at 25°C without shaking. Several flasks of P110 were harvested together to give a total wet weight of mycelium equivalent to the wet weight of a single pad of ST 74A. Amino acids were extracted by placing the mycelium in 5 ml boiling water for 10 minutes. Debris was removed by centrifugation and the supernatants were evaporated to dryness. Each sample was dissolved in 0.5 ml distilled water. Amino acids present in a 10 µl aliquot of the concentrated sample were separated by high voltage electrophoresis (6.7 per cent formic acid, pH 1.6, 105 minutes at 7800 volts). The separated amino acids were then separated by descending chromatography in 77 per cent ethanol.


Electrophoresis 105 min, 7800 volts, pH 1.6
Origin
X1
X3
X2
ASP
GLU
GLN
ARG
LYS
HIS
GLY
HSER
SER
THR
MET
ALA
ILEU
VAL
P110
Chromatography in 77% EtOH


Electrophoresis 105 min, 7800 volts, pH 1.6
Origin
X1
X3
ASP
GLU
GLN
ARG
LYS
HIS
GLY
SER
THR
PHE
MET
ALA
ABU
VAL
LEU
ST74A
Chromatography in 77% EtOH

Hydrolysis of the amino acid extract of P110 results in the formation of a compound which migrates to the location of homoserine lactone. The unknown compound and homoserine lactone prepared by acid hydrolysis of commercially-prepared homoserine give a yellow spot after staining with the cadmium-ninhydrin reagent, whereas most amino acids produce a purple product. Proline also produces a yellow product, but proline is easily distinguished from homoserine lactone by its electrophoretic mobility. Homoserine lactone is similar to arginine in its electrophoretic mobility under the conditions used in these experiments. Finally, the spot corresponding to the unknown compound in an unhydrolyzed sample was cut from an unstained sheet after electrophoresis and was placed on an agar plate containing Vogel's medium and 0.2 mg/ml L-threonine. The paper was removed after 5 minutes. Conidia of the homoserine-requiring strain 51504 grew on the plate in the region where the spot had been eluted. An identical test was used to identify homoserine accumulated by the methionineless mutant studied by Fling and Horowitz (79). This mutant also accumulates threonine. Threonine is required to allow growth of the mutant on limiting amounts of homoserine. Thus the unknown compound accumulated by P110 has been identified as homoserine. The identification of homoserine in P110 supports the hypothesis that the serine requirement has interfered with methionine biosynthesis in the mutant, consistent with the known pathway of methionine biosynthesis in Neurospora (see Figure 21) (80).

The changes in the amino acid pools of P110 might conceivably result from the activity of the L-amino acid oxidase produced by the

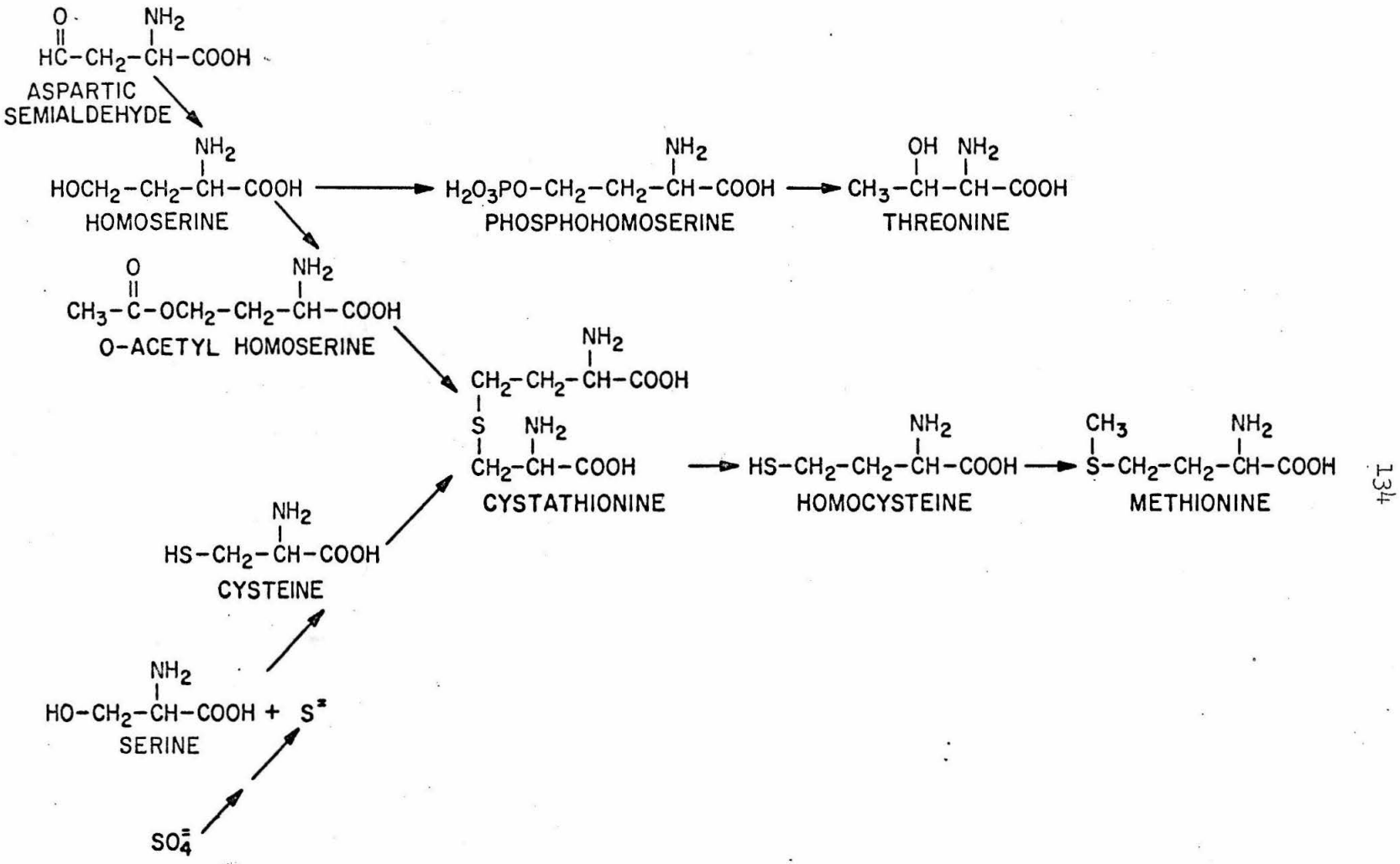


Figure 21. PATHWAY OF METHIONINE BIOSYNTHESIS IN NEUROSPORA

mutant. To test this possibility, the amino acid pools of P110 were extracted from shaken cultures, which do not produce L-amino acid oxidase. Cultures of P110 and ST 74A were grown to 35-40 mg dry weight on a reciprocal shaker at 25°C. No L-amino acid oxidase was observed in duplicate cultures. The accumulation of threonine and the decrease in alanine is still observed in shaken cultures, as shown in Table XXIII. The concentration of glycine in shaken cultures of ST 74A has declined to a level only slightly greater than the concentration of glycine in P110. Compounds migrating to the region of serine are accumulated to a lesser extent in shaken cultures of P110 than in standing cultures, but the difference between P110 and ST 74A is still significant. Aspartic acid and leucine are still present in higher concentration in P110 than in ST 74A, while glutamic acid is significantly lower in P110 than in ST 74A. These results indicate that the changes in the amino acid pools of P110 do not result from the presence of L-amino acid oxidase in the mutant. Rather, the changes in the amino acid pools of P110 appear to reflect the primary biochemical lesion in the mutant. The low glycine and serine pools in P110 and the accumulation of threonine and homoserine by the mutant are consistent with the hypothesis that P110 is blocked in the production of serine and/or glycine.

TABLE XXIII

A comparison of the amino acid pools in P110 and ST 74A grown with shaking

Amino acid	P110 Set I µmoles/gm	P110 Set II µmoles/gm	P110 Av. µmoles/gm	74A Set I µmoles/gm	74A Set II µmoles/gm	74A Av. µmoles/gm
Alanine	35.29	30.34	32.82	162.66	206.70	184.68
Arginine	40.72	37.87	39.30	25.62	28.96	27.29
Aspartic acid	7.31	8.26	7.78	3.14	3.51	3.32
Glutamic acid	19.91	20.36	20.14	90.18	83.77	86.98
Glutamine	52.91	51.06	51.98	56.09	59.02	57.56
Glycine	4.61	4.48	4.54	5.76	6.87	6.32
Histidine	7.82	7.75	7.78	2.72	3.46	3.09
Isoleucine	7.69	7.03	7.36	--	--	--
Valine	--	--	--	9.83	11.96	10.90
Leucine	3.55	3.52	3.54	1.00	1.34	1.17
Lysine	39.45	36.97	38.21	21.31	21.40	21.36
Methionine	9.87	9.16	9.52	15.63	18.17	16.90
Phenylalanine	--	--	--	5.98	6.58	6.28
"Serine"*	25.21	28.77	26.99	9.90	10.28	10.09
Threonine	40.88	40.74	40.81	3.25	3.30	3.28

TABLE XXIII (continued)

Cultures were grown in 20 ml Vogel's minimal medium and 2 per cent sucrose in 125 ml Erlenmeyer flasks on a reciprocal shaker at 25°C until they had attained a dry weight of 30-40 mg (dry weight of extracted mycelium). Amino acids were extracted in 10 ml boiling distilled water for 30 minutes. Debris was removed by centrifugation and the supernatants were evaporated to dryness. Each sample was dissolved in 0.5 ml distilled water. Amino acids present in a 20 µl aliquot of the concentrated sample were separated by high voltage electrophoresis. Estimates of the concentrations of amino acids were made as described in the text.

*The figures given as "serine" concentrations include compounds other than serine which are accumulated by P110.

Production of L-Amino Acid Oxidase by Wild-type Strains Grown on Threonine or Homoserine

The presence of high threonine and homoserine pools in P110 suggested that the mutant might be induced to make L-amino acid oxidase by these amino acids. Previous studies indicated that adding amino acids to biotin-deficient or nitrogen-deficient medium induced L-amino acid oxidase production by wild-type cultures (2,3). The data shown in Table XXIV indicate that amino acids added to standard Vogel's medium (high biotin, high NH_4NO_3) also induce the production of L-amino acid oxidase by wild-type strains. The amount of enzyme activity induced by amino acids in the presence of high biotin and inorganic nitrogen is less than that induced when the medium is deficient in these compounds, but the increase in activity is significant. Threonine or homoserine induces approximately the same level of L-amino acid oxidase as does aminobutyric acid under these conditions. This result was unexpected, because threonine was not a substrate for the L-oxidase under the conditions of assay used by Thayer and Horowitz (2), while aminobutyric acid was an especially good substrate. The activity of Neurospora L-amino acid oxidase towards homoserine has not been investigated, but homoserine is a good substrate for Crotolus adamanteus L-amino acid oxidase (81). A possible explanation for the induction of L-oxidase by threonine would be that some product of threonine metabolism is the true inducer.

L-aminobutyric acid or DL-homoserine decrease the dry weight of the cultures compared to growth on unsupplemented medium, but L-threonine

TABLE XXIV

Production of L-amino acid oxidase by wild-type strain ST 74A grown on threonine or homoserine

Medium	Dry wt (mg)	L-oxidase (ECU/gm)
Vogel's	28.6	0.20
Vogel's + 0.5 mg/ml L-aminobutyric acid	14.4	1.55
Vogel's + 0.2 mg/ml L-threonine	24.9	0.75
Vogel's + 2.0 mg/ml L-threonine	21.4	1.20
Vogel's + 0.4 mg/ml DL-homoserine	3.6	1.36
Vogel's + 4.0 mg/ml DL-homoserine	4.7	1.47
N-free Vogel's + 0.5 mg/ml L-amino-butyric acid*	--	6.45

Cultures were grown in 20 ml medium in 125 ml Erlenmeyer flasks for 48 hours at 25°C without shaking. All values given are averages of duplicate cultures. Duplicate cultures were in close agreement.

*Grown for 80 hours. The dry weight of duplicate cultures was not determined.

was only slightly inhibitory. Severe inhibition of growth does not appear to be a prerequisite for L-amino acid oxidase induction by amino acids.

DISCUSSION

Synthesis of L-Amino Acid Oxidase and NADase by P110

One of the two major questions posed in this thesis is why P110 synthesizes L-amino acid oxidase and NADase while growing on minimal medium. The results of genetic studies indicated that constitutive synthesis of NADase is separable from constitutive synthesis of L-amino acid oxidase. The finding of both characters in a single mutant which arose after uv-irradiation of ST 74A may be fortuitous. Constitutive synthesis of NADase in the absence of L-amino acid oxidase has been observed in strains carrying "fluffy," a gene which prevents the production of macroconidia by a culture (7,83). Inheritance of constitutive synthesis of NADase appears to be complex and was not pursued in the studies of P110 beyond the separation of this character and the constitutive synthesis of L-oxidase.

Poor growth on minimal medium and constitutive synthesis of L-oxidase are inherited as a single gene (designated P110) on linkage group IV near the centromere. Attempts to determine whether P110 is dominant or recessive in heterocaryons were inconclusive, apparently due to unexpected fluctuations in the ratio of component nuclei in the heterocaryon.

L-amino acid oxidase is not present in conidia of P110. Synthesis of the enzyme in P110 grown without shaking on minimal medium begins during the lag period preceding the onset of rapid growth, and reaches a maximum specific activity (ECU/gm wet weight of mycelium)

at the end of the lag period. Early in the investigation of P110, the correlation between high L-oxidase activity and the increased growth rate of the mutant suggested a causal relationship between enzyme synthesis and growth. For example, the L-oxidase might circumvent a blocked metabolic pathway. However, several observations indicate that L-oxidase is not indispensable for growth of P110 on minimal medium. Synthesis of L-oxidase can be prevented by adding Tween 80 or one of several keto acids to the medium, without inhibiting the growth of P110. Growing P110 in shaking cultures prevents the synthesis of L-oxidase and markedly improves the growth of the mutant.

An alternative explanation for the synthesis of L-amino acid oxidase by P110 during the lag period prior to rapid growth is that inhibition of growth _per se_ results in derepression of the oxidase. Burton considered this explanation for the correlation between slow growth and high L-oxidase activity seen in some strains, but discarded it as a general rule when he found that limiting the growth of mutant CMI 17836 _inos_ by providing a suboptimal amount of inositol decreased the oxidase activity per unit weight of mycelium (3). Studies concerning the growth requirement of P110 and its relationship to L-oxidase production also argue against this simple hypothesis.

The identification of serine or glycine as the compound required by P110 suggested that one of these amino acids might specifically repress the L-oxidase although the L-oxidase is not known to be involved in the biosynthesis of serine. In support of this hypothesis was the finding that the addition of L-serine to minimal medium reduces the rate

of L-oxidase synthesis in P110, although the rate of L-oxidase synthesis in P110 grown on serine is not reduced to the fully repressed level observed in wild-type cultures. A change in the repressed level of enzyme synthesis in derepressed mutants has been noted by others. For example, mutants which produce derepressed levels of histidine biosynthetic enzymes in the presence of histidine were observed to produce even greater amounts of the enzymes when the amount of histidine in the medium was reduced (84). Ames and Hartman concluded that the mutants were still under histidine control, but that the repressed level is higher in the mutants than in wild-type cultures. Metzenberg also observed that mutant strains which produce derepressed levels of invertase and trehalase in the presence of mannose at 35°C are repressed by mannose at 23°C, but the repressed level is much higher than that in wild-type (85).

That the decrease in rate of enzyme synthesis in P110 grown on serine is not due to an increased rate of growth is shown by the fact that glycine allows the same rate of growth as serine, but glycine does not reduce the rate of L-oxidase synthesis by P110.

Two serineless mutants which are genetically distinct from each other and from P110 also produce large amounts of L-oxidase when grown on limiting serine. Several other amino acid auxotrophs investigated during the present study produce L-oxidase under growth-limiting conditions, but not all auxotrophs share this property. Apparently, limiting the rate of growth of a culture is not sufficient to derepress the L-oxidase, in agreement with the observations of Burton (3).

A case involving synthesis of enzymes which appear to be unrelated to the biochemical lesion in the mutants under study has been reported previously. Carsiotis and Lacy observed increased activity in both tryptophan synthetase and indole glycerol phosphate synthetase in all known histidine mutants of Neurospora (86). The basis for the increased enzyme levels was not elucidated, but a number of possibilities were eliminated. Among those discarded was the possibility that limitation of growth caused increased production of the tryptophan biosynthetic enzymes. Wild-type starved for carbon or adenineless mutants starved for adenine produced normal levels of the enzymes. Certain other auxotrophic strains showed similar high activities of the tryptophan biosynthetic enzymes, but the authors pointed out that in contrast to the histidineless strains, non-isogenic strains were being compared in this case.

L-amino acid oxidase synthesis by certain auxotrophs under growth-limiting conditions may be related to either one or both of the conditions which are associated with L-oxidase production by wild-type cultures. In one case, the enzyme appears to be induced by amino acid substrates, while in the second case, the L-amino acid oxidase is derepressed. The studies of Burton (3) and Thayer and Horowitz (2) indicated that L-oxidase was induced by the addition of casamino acids to medium containing suboptimal amounts of biotin. A similarity between induction and production of L-oxidase by auxotrophs is that shaking reduces the amount of L-oxidase produced in both cases. Wild-type cultures are induced to synthesize L-amino acid oxidase by growth

on L-α-amino-n-butyric acid as the sole source of nitrogen in a high biotin medium. Tyrosinase is not found in these induced cultures, nor in the amino acid auxotrophs which produce oxidase while growing on limiting amounts of substrate.

Aminobutyric acid added to standard Vogel's medium (high nitrogen and high biotin) induces L-amino acid oxidase in wild-type cultures, but the amount of L-oxidase induced is less than that produced if aminobutyric acid is added to nitrogen-free medium. It is also less than the amount of L-amino acid oxidase produced by P110 grown on minimal medium, but significantly more L-oxidase than the basal level observed in wild-type cultures grown on minimal medium. Adding threonine or homoserine to standard Vogel's medium also induces L-oxidase production by wild-type, suggesting that production of L-amino acid oxidase by P110 grown on minimal medium may result from induction by the high threonine and homoserine pools present in the mutant. The difference in the magnitude of L-oxidase present in P110 compared to wild-type grown in the presence of threonine or homoserine may reflect added effects due to the mutant's starvation for serine and glycine. If P110 is induced by its accumulation of threonine and homoserine, then one might postulate that shaking the cultures reduces the level of L-oxidase induction by reducing this accumulation. Contrary to this hypothesis is the observation that the level of threonine remains high in shaken cultures of P110. How shaking prevents the production of L-oxidase in induced cultures and in the auxotrophs is not known.

The suggestion that changes in the amino acid pools of an organism may affect the regulation of enzymes has been made previously. Davis observed that production of derepressed levels of ornithine transcarbamylase (OTC) by an exotic strain grown in the presence of arginine resulted from a gene which severely limits the size of the intracellular arginine pool (87). The presence of arginine in the medium did not repress OTC because the intracellular pool of arginine remained small.

In addition to conditions in which amino acids induce synthesis of L-amino acid oxidase both tyrosinase and L-amino acid oxidase are derepressed in wild-type cultures by a variety of growth-inhibiting conditions. If a culture is transferred from minimal medium to phosphate buffer, both of the enzymes are observed to increase in parallel (8). The addition of antimetabolites such as ethionine, D-phenylalanine, or cycloheximide to cultures grown on subminimal medium (41) derepresses both enzymes in most cultures. Horowitz postulated that antimetabolites reduce the rate of synthesis of a protein repressor or repressors regulating the production of tyrosinase and L-amino acid oxidase (88).

That the derepressible L-amino acid oxidase is identical to the induced enzyme was shown by comparing the properties of partially purified L-oxidase produced under the two conditions. The oxidases were indistinguishable with respect to size as judged by gel filtration, and they had identical thermostabilities.

If L-oxidase is synthesized by P110 as the result of derepression, then the absence of tyrosinase in the mutant requires explanation.

If L-oxidase and tyrosinase production are controlled by separate repressors, then some conditions might reduce the concentrations of both repressors (giving an apparent coordinate control of the enzymes), while other conditions might affect only one of the repressors. Starvation for serine or another required metabolite may reduce the rate of synthesis of the L-oxidase repressor without affecting the tyrosinase repressor. Conceivably, those auxotrophs which synthesize L-oxidase when starved for their requirement may be deficient in amino acids which are present in large amounts in the L-oxidase repressor.

Alternatively, derepression of tyrosinase may require an internal effector which is not required for derepression of L-oxidase. Gorini has observed that derepression of the arginine biosynthetic pathway enzymes in E. coli will not occur in the absence of glutamate, the substrate for ornithine biosynthesis (79). It may be significant that neither tyrosine nor phenylalanine is found in the intracellular free amino acid pool of P110 grown on minimal medium.

An hypothesis which is equally consistent with existing data is that a single apo-repressor binds to different co-repressors to control the structural genes for tyrosinase and L-oxidase. Serine, for example, could be a co-repressor of L-oxidase formation, without affecting repression of tyrosinase. Conditions which reduce the concentration of the apo-repressor (e.g. addition of cycloheximide) would derepress both enzymes, while conditions which affect just one of the co-repressors (P110?) would affect only one of the two enzymes. Induction of L-oxidase by amino acids may result from competition between

added amino acids and the co-repressor(s) of L-oxidase synthesis, so that induction would be a special case of the reversal of repression.

Each of the models proposed to explain the constitutive synthesis of L-amino acid oxidase by P110 conforms to the hypothesis that oxidase synthesis is controlled by repression. The data are equally consistent with the proposition that derepression of L-oxidase in P110 is the direct result of the mutant's serine requirement, or that oxidase production is an indirect consequence of serine deficiency.

The Amino Acid Requirement of P110

The second problem investigated in this thesis was the nature of the biochemical lesion in P110 which results in its serine or glycine requirement.

The conclusion that P110 is a "leaky" auxotroph which requires serine or glycine for optimum growth comes from several lines of evidence. P110 reaches the same final dry weight in the presence or absence of (serine) supplement, a characteristic used by Bonner et al. to define "leaky" mutants (24). Serine and glycine were identified as two components of yeast extract which supported the growth of P110. The ready interconversion of serine and glycine in Neurospora was demonstrated by Sojka (77). P110 was also found to be qualitatively similar to all other known serine-requiring strains of Neurospora.

The identification of the step in serine biosynthesis blocked in P110 would seem to be a straightforward problem in biochemistry. Sojka and Garner had published evidence for two pathways of serine biosynthesis

(see Figure 1) in Neurospora (25), and it seemed reasonable that one of the enzymes involved in these pathways would be defective in P110. Each of the enzymes identified by Sojka and Garner was prepared from P110 and compared to the comparable enzyme prepared from ST 74A. No differences which can account for the serine requirement of P110 were found between the activities of the serine biosynthetic enzymes in P110 compared to ST 74A.

Sojka tried to identify the blocked reaction in each of the other known serineless strains of Neurospora. Both ser-2 and ser-3 showed ten per cent of the wild-type activity of phosphoserine transaminase. Mutant ser-3 also showed a reduction in serine transaminase activity, while ser-2 showed a 3X increase in the activity of serine transaminase (77). Whether phosphoserine transaminase and serine transaminase activity are due to a specific transaminase is unknown (25). Under the reaction conditions used in the studies reported in this thesis, the transaminase activities were rather low (phosphoserine transaminase activity was 7.9×10^{-3} μmoles glutamate formed/mg protein/hour in ST 74A). The possibility that the phosphoserine transaminase reaction may be catalyzed by a transaminase with rather broad specificity has not been eliminated in bacteria (29). Serineless mutants of E. coli and S. typhimurium have been found for each of the other steps in serine biosynthesis (29). Sojka found no differences in the specific activity of any of the serine biosynthetic pathway enzymes in either H 605 ser-1 or C 127 ser-1 (77). Thus the ser-1 mutants are similar to P110 with respect to the wild-type activities observed for each of the enzymes

implicated in serine biosynthesis by the pathways described by Sojka and Garner. P110 maps on linkage group IV, while ser-1 maps on linkage group III, indicating that the two mutants involve separate genes.

An analogous result was obtained by Wagner et al. during a study of mutants blocked in the synthesis of isoleucine and valine (89, 90). In this case, enzymes extracted from mycelium showed high activity, despite evidence from the accumulation of intermediates and feeding experiments that the enzymes were inactive in vivo. The explanation for this paradox appears to be that enzyme activity within the cell requires a particular arrangement of the isoleucine-valine biosynthetic enzymes within an aggregate (90). The importance of organization of enzymes, particularly with respect to their interaction with the structural components of the cell, has been emphasized by the work of Woodward and Munkres (91). These investigators found that mutant forms of malate dehydrogenase have wild-type affinities for malate when free in solution, but different affinities for malate when complexed to the mitochondrial structural protein.

Whether the explanation for the serine requirement of P110 and ser-1 involves organization of the serine biosynthetic enzymes into an aggregate or interaction with structural protein is unknown. The serine biosynthetic activities are assayed in the soluble enzyme fraction, but isoleucine and valine biosynthetic enzymes are released into this fraction by similar handling of the mycelium. In the absence of evidence for the accumulation of intermediates by the mutants or evidence for the cross-feeding of one mutant by the other [such attempts

have all given negative results (25,77)], no particular enzyme in the reaction sequence is suggested for more intensive study. An attempt to study the physical properties of each of the serine biosynthetic enzymes under various conditions with the hope of identifying the altered enzyme was beyond the scope of this work.

An alternative explanation for the results with P110 and ser-1 is that the two pathways studied by Sojka and Garner do not exhaust the sources of serine in Neurospora. Evidence that serine may be derived from glycine was given by Sojka (77) and confirmed by me. Abelson and Vogel obtained evidence from labeling studies that radioactivity from C^{14}-glycine is incorporated into serine and cysteine (92). That glycine may be derived from glyoxylate (Figure 2) in Neurospora was first shown by Wright (34,35). Combépine and Turian were unable to grow ser-1 on glyoxylate and concluded that this pathway must not play a major role in glycine synthesis in Neurospora (37). The difference in the results obtained by Wright on the one hand and Combépine and Turian on the other is explained by the difference in the carbon source used by the two investigators. None of the serine-glycine auxotrophs will utilize glyoxylate in the presence of sucrose (as used by Combépine and Turian), whereas all of them use glyoxylate added to glycerol minimal medium (as used by Wright). Whether sucrose blocks the uptake or utilization of glyoxylate was not determined.

Sjogren and Romano have shown that Neurospora's isocitrate lyase, which splits isocitric acid to form succinic acid and glyoxylic acid, is repressed by glucose (93), suggesting that the glyoxylate

pathway is subject to catabolite repression (94). Flavell has observed derepression of the enzymes of the glyoxylate shunt after transfer of cultures from sucrose medium to one in which acetate was the sole carbon source (95). Isocitrate lyase activity is high in conidia (93), and may provide glyoxylate for glycine synthesis during early growth. That glycine may be the specific requirement of P110 is suggested by the finding that glycine allows P110 to reach the same final dry weight as ST 74A, the wild-type strain from which P110 was derived. No concentration of serine allowed P110 to reach a final dry weight greater than that attained on minimal medium, which is approximately 2/3 the dry weight attained by ST 74A.

In addition to the pathways which have been discussed for glycine biosynthesis, another source of glycine may be threonine. The cleavage of threonine to yield glycine and acetaldehyde was first described by Braunstein and Vilenkina (96). An enzyme which catalyzes this reaction has been isolated from a variety of mammalian tissues and is called threonine aldolase, although allothreonine is a better substrate for the partially purified enzyme than is threonine (97). E. coli will utilize exogenous threonine to supply glycine, but endogenous threonine does not appear to be metabolized in this way (98). The amount of glycine formed from exogenous threonine is inadequate to support rapid growth of most serine-glycine auxotrophs of E. coli, but Van Lenten and Simmonds obtained a mutant which could grow on serine, glycine, or threonine, designated S/G/T (99). Utilization of threonine in this case is attributed to an enhanced ability to effect a net

synthesis of glycine from threonine, perhaps due to decreased threonine dehydrase activity in the mutant. The cleavage of threonine to glycine in the S/G/T mutant does not appear to involve the formation of acetaldehyde, according to isotopic labeling studies (99).

Using uniformly labeled C^{14}-glucose in an *in vitro* *Neurospora* system, Sojka and Garner observed labeling of threonine, serine, glycine, and alanine (25). Using the method of isotope competition in this *in vitro* system, the glycine formed was found to be related to threonine (i.e. the incorporation of counts into glycine was reduced by adding unlabeled threonine, and not by adding unlabeled serine). The labeling studies of Abelson and Vogel, however, do not support the hypothesis that glycine is derived from endogenous threonine in *Neurospora crassa* (92). Counts introduced in uniformly labeled aspartic acid were found in protein, aspartic acid, methionine, threonine and isoleucine, but not in glycine. These studies were performed in vigorously agitated cultures grown on glucose, however, and the possibility remains that different pathways for glycine biosynthesis may be utilized under different conditions of culture. Consistent with this suggestion was the finding by Wagner and Bergquist that glycine and acetaldehyde were formed from exogenous threonine by a strain of *Neurospora* which is inhibited by threonine (78). Apparently, *Neurospora* can carry out this reaction under certain conditions.

In *E. coli*, the relationship between glycine and serine is altered by the carbon source. Serine is the precursor of glycine in the

presence of glucose, but exogenous glycine is a precursor of serine when either fructose or acetate is the energy source (98).

A block in the synthesis of glycine from threonine in P110 would account for the high accumulation of threonine in the mutant. Alternatively, threonine may accumulate as the result of the reduced incorporation of homoserine into cystathionine. Reduced incorporation of homoserine into cystathionine results from a deficiency of cysteine produced from serine.

The accumulation of threonine in P110, regardless of its origin, may account in part for the pleiotropic effects of the P110 mutation. An antagonism between threonine and homocysteine, methionine, thiamin, serine and adenine can be inferred from the properties of the threonine-sensitive strain of Neurospora (100). In the presence of high threonine, glycine cannot replace the serine requirement induced in the mutant (101). Threonine has been shown to antagonize the utilization of serine by serine-requiring mutants of Lactobacillus delbruckii (102).

An explanation for the properties of P110 not yet discussed is that the mutant may be defective in the metabolism of active "C_1" units. Serine has been implicated as the reservoir for "C_1" units in yeasts (33) and may well serve that function in Neurospora. If the production of "C_1" units from other sources were blocked in P110, serine might be depleted by a channeling into reactions requiring the "C_1" unit. P110 did not respond to sodium formate or formaldehyde added to minimal medium, but the hypothetical block in "C_1" metabolism could be far removed from activation of these compounds.

Finally, the relatively crude purification procedure which identified serine and/or glycine as the active factors in yeast extract does not eliminate the possibility that components of yeast extract other than serine or glycine may be required by P110.

To locate the biochemical lesion in P110, extensive studies involving metabolism of uniformly labeled glyoxylate, threonine, serine and glycine in both P110 and wild-type are indicated. As in E. coli, the interrelationships among these compounds may be complex and variable, depending on cultural conditions, age of the culture, or other unknown factors. An indication of the known pathways related to serine biosynthesis is given in Figure 22.

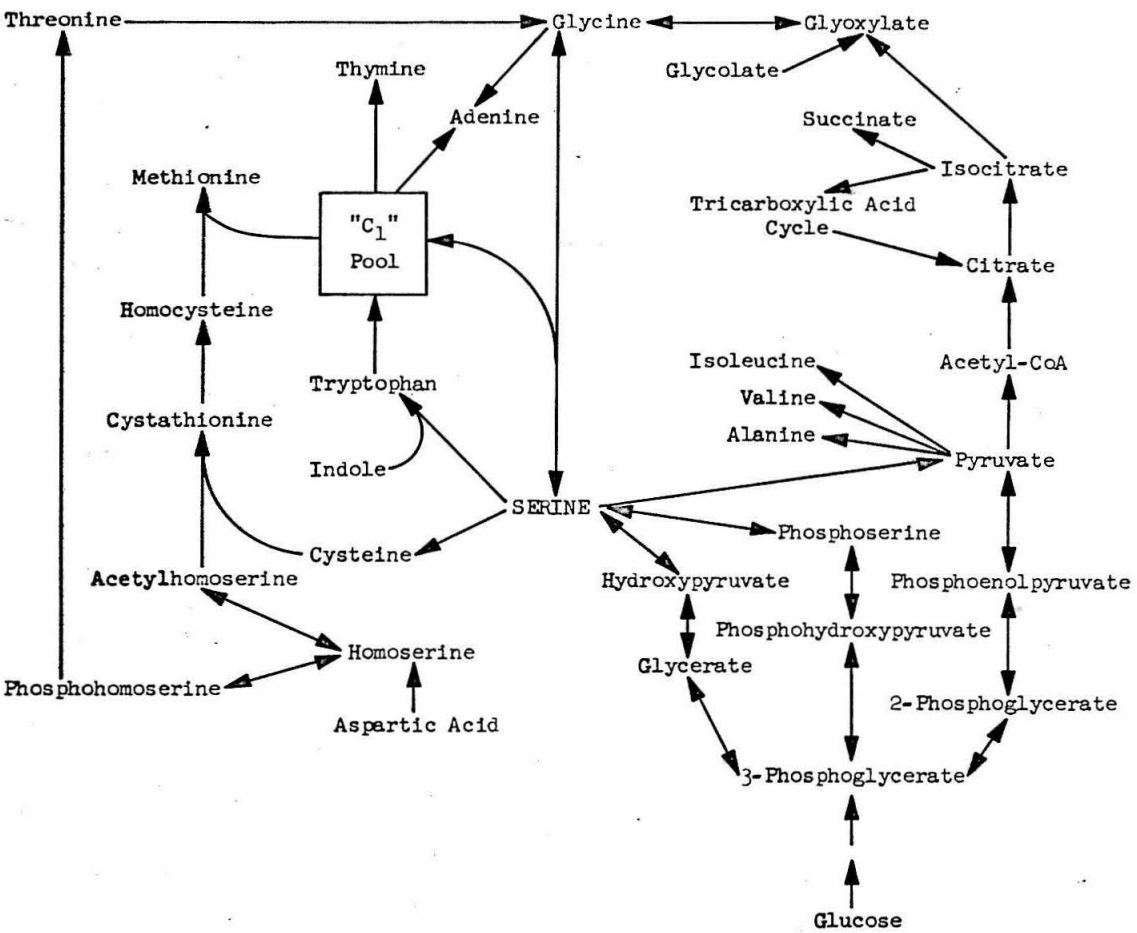


Figure 22. Biochemical Pathways Related to Serine

APPENDIX

An Attempt to Isolate Mutants Deficient in L-Amino Acid Oxidase

Mutants deficient in L-amino acid oxidase were sought in order to locate the L-oxidase structural gene.

L-amino acid oxidase appears to be a non-essential enzyme for growth of wild-type cultures on minimal medium. Mutant hunts were performed under conditions which made the L-amino acid oxidase essential for growth. Mutagenized conidia were plated on medium which contained L-α-amino-n-butyric acid as the sole nitrogen source. Aminobutyric acid can be transaminated by crude extracts of Neurospora (104), but the amount of ammonia obtained in this way is insufficient to support the growth of mutants deficient in glutamic acid dehydrogenase (105). In theory, growth of a culture on aminobutyric acid as the sole nitrogen source should require deamination of the amino acid by L-amino acid oxidase. In support of this hypothesis, L-amino acid oxidase has been observed in all strains which have grown on aminobutyric acid as the sole nitrogen source. Of particular interest was the observation that the mutant ty-1, which produces no L-amino acid oxidase or tyrosinase when starved in phosphate buffer (43) produces L-amino acid oxidase when grown on aminobutyric acid as the sole nitrogen source (see Table XXV). Tyrosinase is not produced by ty-1 under these conditions. Similarly, T-22, a mutant which cannot be derepressed to produce tyrosinase and L-amino acid oxidase by any of the known methods, produces

TABLE XXV

Synthesis of L-amino acid oxidase by cultures growing on aminobutyric acid as the sole nitrogen source

Culture	Nitrogen source	Dry wt (mg)	L-oxidase	Tyrosinase
			ECU/gm	ECU/gm
II6a;cot	NH_4NO_3	35.3	3.3	0
II6a;cot	aminobutyrate	35.0	8.0	0
ty-1	NH_4NO_3	110.0	0.57	0
ty-1	aminobutyrate	46.8	3.3	0

Cultures were grown without shaking for 7 days at 25°C on 20 ml medium in 125 ml Erlenmeyer flasks. Ammonium nitrate was supplied in standard Vogel's medium; aminobutyrate (0.5 mg/ml) was added to N-free Vogel's medium. The carbon source was 2% sucrose in both cases.

large quantities of L-amino acid oxidase when grown on aminobutyrate (46).

Most mutant hunts utilized the L-amino acid oxidase constitutive strain, P110, to eliminate the isolation of mutants blocked in induction of L-amino acid oxidase by aminobutyric acid. A sexual reisolate of P110 which grows colonially at elevated temperatures (II6a;cot) was used to facilitate examination of large numbers of conidial isolates. Conidia were irradiated with 50KV, 30ma x-rays at a distance of 7 centimeters from the x-ray window for 2 minutes (100,800r), giving approximately 3% survival. Irradiated conidia were plated on aminobutyric acid as the sole nitrogen source, and plates were incubated at 32°C. Cultures which grew as poorly as strains grown on nitrogen-free medium were isolated and retested on liquid medium. Mutants which appeared unable to grow on aminobutyric acid as the sole nitrogen source were tested for L-amino acid oxidase after growth on Vogel's minimal medium. Several hundred possible mutants were isolated from 5 separate mutant hunts using II6a;cot, and 38 possible mutants were isolated from one hunt using C102-15300-4-2A. In all cases, strains isolated by this criterion produced at least as much L-amino acid oxidase as II6a;cot. In the experiments using C102-15300-4-2A, cultures were tested for L-amino acid oxidase production after derepression by cycloheximide. Again, no mutants were obtained.

Why the selection procedure was unsuccessful is unclear. Perhaps the mutation is lethal, or perhaps the rate of mutation is too low for mutants to be detected in the number of cultures examined.

Approximately 1 per cent of the cultures arising from plated irradiated conidia were isolated as possible L-oxidase deficient mutants. Thus, about 50,000 cultures were scanned for the character.

Another explanation for the failure of these hunts is suggested by a report concerning isolation of trehalase mutants in Neurospora (106). Trehalase, like L-amino acid oxidase (7), is an intramural enzyme (i.e. it is located between the cell membrane and the cell wall). These studies indicate that conidia lacking trehalase are fed by products released by wild-type conidia unless precautions are taken to reduce the amount of cross-feeding between conidia on plates. Perhaps the activity of L-amino acid oxidase present in wild-type conidia releases ammonia into the medium which is then used as a nitrogen source by mutants deficient in L-amino acid oxidase. Application of a procedure similar to that described for isolating trehalase mutants may prove rewarding in future efforts to isolate L-amino acid oxidase deficient mutants.

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INTRODUCTION

Multiple electrophoretic forms of tyrosinase were occasionally observed by Sueoka (1) and by Horowitz et al. (2) in crude extracts of genetically homogeneous cultures of N. crassa. This heterogeneity differs from that found in the genetic polymorphism exhibited by exotic strains isolated from different geographical locations (2) in that it occurs sporadically. One of the 2 or 3 separate bands observed in paper electrophoresis always corresponded to the single band of the more usual extracts. The extra bands were very labile. They could not be found in extracts which were partly purified, or which had been allowed to warm up to room temperature, or which had been stored overnight in a refrigerator. Apparently the same phenomenon was observed by Fox and Burnett (3), who presented evidence that the three electrophoretic forms isolated by them are interconvertible. These investigators postulated that the different electrophoretic forms separated by continuous flow paper electrophoresis are identical to the different allelic forms of tyrosinase, T^L and T^S (3). This suggestion has since been withdrawn (4). Horowitz et al. suggested that the unstable components might result from labile associations formed between tyrosinase molecules and other substances in crude extracts (2).

In view of the possible importance of this phenomenon for understanding the genetics and biochemistry of tyrosinase, a study was initiated to examine it in more detail than had been done previously.

MATERIALS AND METHODS

Strains

All strains used during this study were nutritionally wild-type. The strains differ with respect to their tyrosinase alleles. Strains 11736A,T^{L}; 120-1a,T^{Sing-2}; 65-1434A,T^{PR}; 4-137a,T^{L}; 854a,T^{S}; 4-121A,T^{S}; and Sing-2a,T^{Sing-2}; were all kindly provided by Mrs. Helen Macleod Feldman. Extensive use was made of strain III-70a,T^{PR}, which was derived as a single spore isolate from a cross between 4-137a,T^{L} and 65-1434A,T^{PR}.

Cultures were maintained as described in Part I (pp. 15 and 16).

Derepression of Cultures

For most experiments, cultures were derepressed by a slight modification of the Pall method (5). Cultures were grown on 1/2X Vogel's salts and 1/2 per cent sucrose for 48 or 72 hours. Derepression was initiated by adding 2 mg DL-ethionine or 2 mg D-phenylalanine to each flask. Incubation was continued for an additional 48 hours on a reciprocal shaker (80 strokes/minute) at 25°C. In a few experiments, an identical procedure was used, with the exception that cultures were grown on 1X Vogel's salts and 1/2 per cent sucrose.

An alternative method of derepression used pads grown on 1X Vogel's salts and 2 per cent sucrose for 48 hours. Each pad was washed twice with 20 ml sterile distilled water, which was carefully decanted after each wash. The washed pad was then suspended on 5 ml sterile

carbon-free Vogel's medium containing 0.2 mg/ml DL-ethionine, or on 5 ml 0.02M sodium phosphate buffer, pH 6. In some experiments, 0.2 mg/ml DL-ethionine or 0.2 mg/ml D-phenylalanine was included in the phosphate buffer. Cultures were incubated without shaking for an additional 48 hours at 25°C and in the dark.

Tyrosinase was also derepressed by growing cultures on low sulfur medium (6). This medium was prepared by adding 16 μg/ml $MgSO_4 \cdot 7H_2O$ to sulfur-free Vogel's salts. Cultures were grown on 20 ml medium containing 2 per cent sucrose in 125 ml Erlenmeyer flasks at 25°C for 5 days, stationary and in the dark.

The extraction and assay of tyrosinase were carried out as described in Part I (pp. 17 and 19).

Paper Strip Electrophoresis

Paper strip electrophoresis was carried out in 0.05M sodium phosphate buffer, pH 6, containing 0.1 per cent bovine serum albumin (Bovine Albumin Powder. Fraction V. Armour Pharmaceutical Company, Illinois). The paper strips were wetted with 1 per cent serum albumin prior to application of the enzyme extracts. A 10 to 20 μl sample of crude extract, containing approximately 0.25-0.5 ECU of tyrosinase was applied to each strip, using a Beckman Sample Applicator (cat. no. 320005). A constant current of 1.25 ma per strip was maintained for 19-24 hours in a Spinco Model R (Durram) Paper Electrophoresis Cell. All electrophoretic runs were performed at 4°C. At the end of the run, the holder containing the strips was spread such that it and the paper

strips were horizontal. The strips were sprayed with a fine mist of 4 mg/ml DL-DOPA in 0.1M sodium phosphate buffer, pH 6. Narrow zones of orange dopachrome identified the position(s) of tyrosinase activity on the strips.

Zone Electrophoresis in a Density Gradient

Zone electrophoresis was performed in a Column Electrophoresis Apparatus Type LKB 3340C, utilizing an LKB Power Supply 3371C (110 volts, 60 cys). Electrophoresis was carried out in cold 0.05M sodium phosphate buffer, pH 6, using a linear sucrose gradient (0-20 per cent sucrose). Samples were applied to the density gradient at a location about 1/3 of the distance down the length of the column (sucrose concentration approximately 7%). The density of the column at this location was determined, and the sample density was adjusted so that the 5 ml sample entered the column as a narrow band (7). A constant current of 30 ma was maintained for 19-20 hours, with a column temperature of 2-4°C. After completion of electrophoresis, 1 ml samples were collected from the base of the column and aliquots of these samples were analyzed for tyrosinase activity.

Continuous Flow Paper Electrophoresis

Electrophoretic runs were carried out in 0.02M sodium barbitol buffer, pH 8.6, in a Spinco Model CP cell at 4°C. In one experiment, the lower curtain (Part No. 400-235) was wetted with 1 per cent bovine albumin (fraction V) before application of the sample and 0.1 per cent

bovine serum albumin was added to the buffer reservoirs. A constant current of 40 ma was applied for 24 or 36 hours. Fractions were collected continuously from the bottom of the paper.

Genetic Analysis

All crosses were performed on Westergaard-Mitchell medium (8), containing 2 per cent sucrose. In cross III (4-137a,T^{L} x 65-1434A,T^{PR}), 4-137a,T^{L} was used as the protoperithecial parent. In all other crosses, strains were coinoculated onto agar plates of crossing medium. Ascospores were isolated onto slants of Horowitz complete medium (9). Spores were heat-shocked for 45 minutes to 1 hour at 56-60°C; thereafter, cultures were incubated at 25°C.

RESULTS

Genetic Analysis

The multiple electrophoretic forms of tyrosinase were observed infrequently during studies of homocaryons under the conditions employed by the Horowitz group (1,2). To affirm that the multiple forms did not result from heterocaryosis, strains which showed this characteristic were crossed, and the tyrosinase produced by cultures arising from single ascospores was analyzed electrophoretically. The results of a cross between 4-137a,T^L, which produces 2 electrophoretically distinct bands of tyrosinase (2 T^L), and 65-1434A,T^{PR}, which produces 3 bands of tyrosinase (3 T^{PR}), are shown in Table I. These results confirm that under the conditions used in these experiments, homocaryotic cultures may produce more than one electrophoretic form of tyrosinase. The electrophoretic patterns observed among the progeny are shown diagrammatically in Figure 1. The locations of the supernumerary bands appear to be determined by the T allele present. That is, the "extra" bands in a T^{PR} strain are distinct from the "extra" bands in a T^L strain. This implies that the supernumerary bands are not determined by additional tyrosinase structural genes which segregate from the T locus. In support of this hypothesis is the observation that T^{PR} strains may produce one to four bands of tyrosinase activity, some of which are not observed in either parental strain. Similarly, some of the T^L progeny produce a third electrophoretic form which is not observed in 4-137a,T^L. Additional evidence that these tyrosinases are

TABLE I

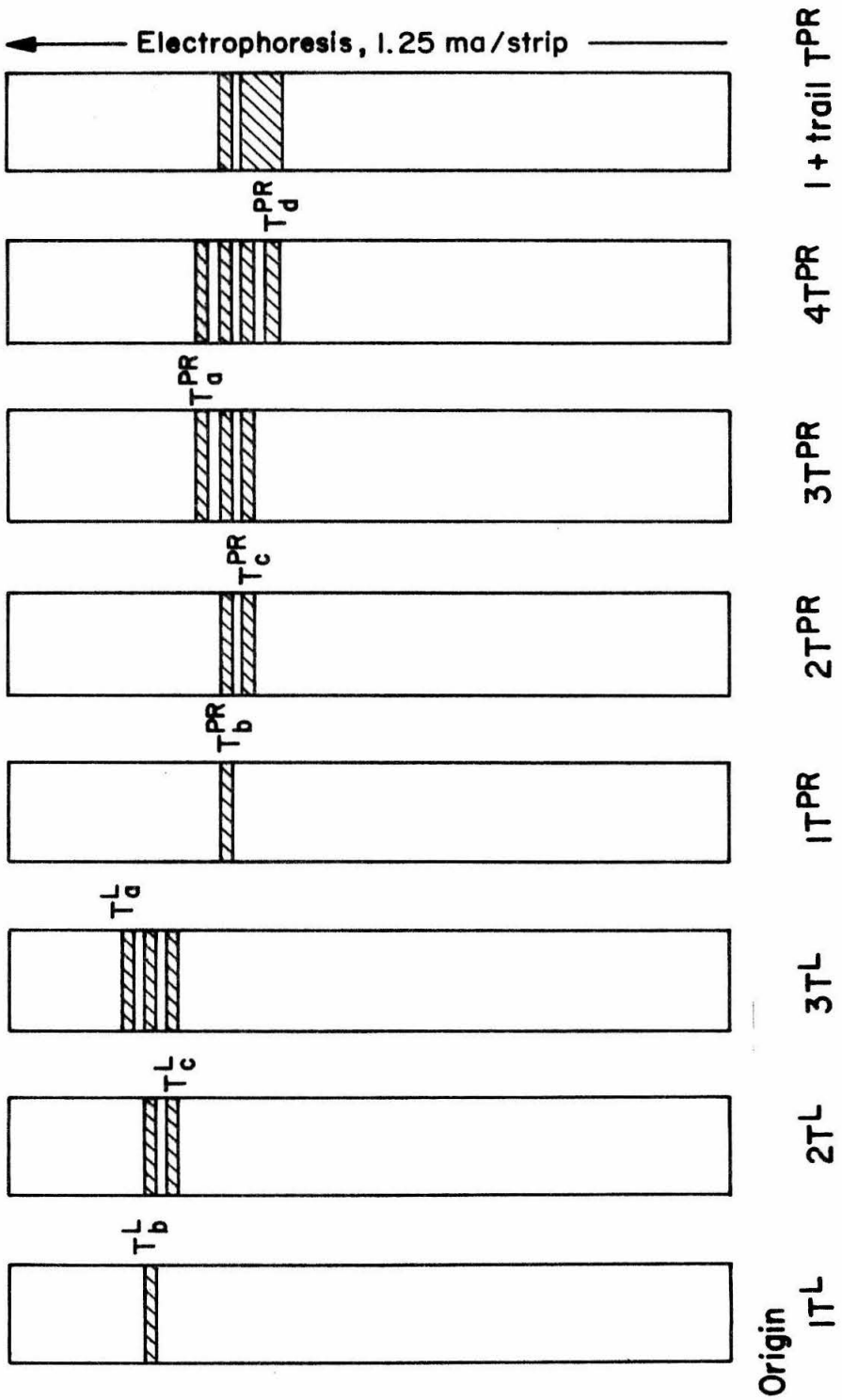
Types of electrophoretic patterns of tyrosinase activity observed among progeny of the cross 4-137a,T^{L} x 65-1434A,T^{PR}

Phenotype*	Number of progeny
3 T^{PR}	17
2 T^{L}	23
2 T^{PR}	14
3 T^{L}	7
4 T^{PR}	3
1 T^{PR}	4
1 + trail T^{PR}	1
	69

*See Figure 1.

Cultures were grown on 1/2X Vogel's salts and 1/2% sucrose in 125 ml Erlenmeyer flasks containing 20 ml medium. Cultures were incubated at 25°C without shaking for 3 days before derepression of tyrosinase was initiated by adding 2 mg DL-ethionine to each flask. During derepression, cultures were incubated on a reciprocal shaker (80 strokes/min) at 25°C for an additional 48 hours. Electrophoretic patterns of crude tyrosinase were determined as described in Materials and Methods. Most cultures were tested in duplicate.

Figure 1. Electrophoretic patterns of tyrosinase activity observed among progeny of the cross 4-137a,T^{L} x 65-1434A,T^{PR}. Crude extracts were applied to paper strips in a Spinco Model R (Durram) Paper Electrophoresis Cell and electrophoresis was carried out as described under Methods.


Electrophoresis, 1.25 ma/strip
Origin
1T^L
2T^L
3T^L
1T^PR
2T^PR
3T^PR
4T^PR
1 + trail T^PR
T^L_b
T^L_c
T^L_a
T^PR_b
T^PR_c
T^PR_a
T^PR_d

all products of the same gene comes from the fact that brief heating (60°C for 2 min) of the extracts results in the disappearance of all the supernumerary bands. The band remaining is identical in position to the band observed in single-banded strains carrying the same T allele. Similarly, this band is the same, whether the extract heated originally contained 2, 3 or 4 bands.

The observation of electrophoretic forms among the progeny which differed from those present in the parental strains suggested that the latter strains might have been heterocaryotic for factors which influence the multiple forms of tyrosinase. The strains used had been maintained by vegetative transfer for many months, and heterocaryosis was not unlikely. Thus, three additional crosses were prepared using freshly isolated progeny from the cross described above. The results of these crosses are given in Table II. Evidently, the variety in the electrophoretic patterns observed among progeny is not reduced by crossing recently isolated homocaryons, as shown by cross VII: III 59A,T^{L} (2 T^{L}) x III 100a,T^{PR} (3 T^{PR}). The small number of patterns observed in cross XII: III 42A,T^{L} (2 T^{L}) x III 100a,T^{PR} (3 T^{PR}) probably results from the small number of progeny analyzed. Most interesting, however, are the results of cross IX: III 28a,T^{L} (2 T^{L}) x III 98A,T^{L} (2 T^{L}), in which only 2 T^{L} progeny were recovered. Although the number of progeny analyzed is small, this cross supports the idea that the positions of the supernumerary bands are restricted by the T allele present, and that the number of these bands is influenced by additional factors. Progeny producing 3 T^{L} bands were observed

TABLE II

Types of electrophoretic patterns of tyrosinase activity observed among progeny of crosses between recently isolated homocaryons

Cross	Progeny phenotype	Number of progeny
VII: 2 T^{L} x 3 T^{PR}	2 T^{L}	50
	3 T^{PR}	28
	2 T^{PR}	15
	3 T^{L}	4
	1 T^{L}	4
	1 T^{PR}	7
	4 T^{PR}	2
		110
IX: 2 T^{L} x 2 T^{L}	2 T^{L}	16
XII: 2 T^{L} x 3 T^{PR}	2 T^{L}	9
	3 T^{PR}	12
	1 T^{L}	2
	1 T^{PR}	1
		24

Experimental procedures as in Table I.

in crosses involving 3 T^{PR} strains, but were not observed in the 2 T^{L} x 2 T^{L} cross.

That non-genetic factors influence the number of electrophoretic bands produced is suggested by the results obtained with ordered asci from the above crosses. These results are shown in Table III. Although some of the asci show Mendelian segregations, several discrepancies are apparent. In particular, ascus 2 and 3 of cross VII and ascus 3 of cross XII contain non-identical sister spores. These results and the spectrum of patterns obtained in the progeny led to an investigation of factors which influence the number of electrophoretic forms of tyrosinase observed in a culture.

The Effect of Method of Derepression on the Tyrosinase Electrophoretic Pattern

The fact that most of the strains examined during the genetic analysis reported above produced multiple electrophoretic forms of tyrosinase, whereas earlier studies revealed few such strains, suggested that the new method of derepression used in these studies was affecting the electrophoretic pattern. To test this suggestion, several strains used during earlier studies (1) were grown up from silica gel stocks (10) and were derepressed by the Pall method (5). As shown in Table IV, each of the stocks except 120-1 $\underline{T}^{Sing-2}$ produced multiple bands under these conditions. The location of the bands obtained with Sing-2a was identical to the positions of T_a^{PR} and T_b^{PR} (see Figure 1). The "major" band of T^{Sing-2} strains is identical in electrophoretic mobility to

TABLE III

Types of asci observed in crosses between strains showing multiple electrophoretic forms of tyrosinase

Cross	Ascus no.	Arrangement of spores
VII	1	3 T^{PR}, 3 T^{PR}, 3 T^{PR}, 3 T^{PR}: 2 T^{L}, 2 T^{L}, 2 T^{L}, 2 T^{L}
	2	2 T^{L}, --, 1 T^{PR}, 3 T^{PR}: 3 T^{PR}, --, --, --
	3	2 T^{L}, 2 T^{L}, 4 T^{PR}, 3 T^{PR}: --, 2 T^{L}, --, --
	4	--, 2 T^{L}, 2 T^{PR}, 2 T^{PR}: --, --, 1 T^{PR}, --
	5	--, --, --, --: 2 T^{L}, 2 T^{L}, 2 T^{L}, 2 T^{L}
XII	1	3 T^{PR}, 3 T^{PR}, 3 T^{PR}, 3 T^{PR}: 2 T^{L}, 2 T^{L}, 2 T^{L}, 2 T^{L}
	2	3 T^{PR}, 3 T^{PR}, 2 T^{L}, 2 T^{L}: 3 T^{PR}, 3 T^{PR}, 1 T^{L}, 1 T^{L}
	3	2 T^{L}, 2 T^{L}, 1 T^{PR}, 3 T^{PR}: 3 T^{PR}, 3 T^{PR}, 2 T^{L}, --

Experimental procedures as in Table I.

TABLE IV

Electrophoretic pattern seen in various strains derepressed by the Pall method

Strain	Tyrosinase activity ECU/gm	Bands
Sing-2a	31.3	2
120-1 $\underline{T}^{Sing-2}$	24.0	1
854 $\underline{T}^{S}$	141	2
4-137 $\underline{T}^{L}$	146	2
11736 $\underline{T}^{L}$	140	2
65-1434 $\underline{T}^{PR}$	128	3

Experimental procedures as in Table I.

T_b^{PR}, the "major" band in T^{PR} strains, in agreement with Horowitz et al. (2). The bands of T^S strains were indistinguishable from the bands of T^L strains in my hands.

Conversely, strains derepressed by the method used during early studies on tyrosinase carried out by the Horowitz group appear to result in extracts displaying a single electrophoretic form of tyrosinase. A strain which had given a highly reproducible 3 T^{PR} pattern when derepressed with ethionine was grown on low sulfur Vogel's minimal medium. This strain called III 70 produced 20.9 ECU of tyrosinase per gram wet weight of mycelium. Paper strip electrophoresis indicated that the extracts from III 70 derepressed in this way contained a single species of tyrosinase, which migrated to the T_b^{PR} position. Similarly, cultures of 4-121 T^S produced 18.5 ECU/gm tyrosinase, which migrated as a single band. Strains 4-137 T^L and Sing-2a produced too little tyrosinase by this method to determine their electrophoretic properties.

The different patterns observed in cultures derepressed by the Pall method and by growth on low sulfur led to an investigation of the tyrosinase electrophoretic patterns obtained from cultures derepressed by each of the various methods used by the Horowitz group. The amount of tyrosinase produced by cultures of III 70 derepressed by each method and the pattern of banding observed is indicated in Table V. The pattern varied considerably with different methods of derepression. The level of tyrosinase activity produced under different conditions also varied considerably, but the data do not support the suggestion that

TABLE V

The effect of different methods of derepression on the tyrosinase electrophoretic pattern and tyrosinase activity in III 70 $\underline{T}^{PR}$

Method of derepression	Tyrosinase activity ECU/gm	Bands
2 mg DL-ethionine added to cultures grown on 1/2X Vogel's salts and 1/2% sucrose for 3 days. Cultures derepressed for 48 hours with shaking.	154	3
2 mg D-phenylalanine added to cultures grown on 1/2X Vogel's salts and 1/2% sucrose for 3 days. Cultures derepressed for 48 hours with shaking.	541	1
Cultures grown on 1X Vogel's salts and 2% sucrose for 3 days. Pad suspended on phosphate buffer for an additional 48 hours, without shaking.	57.1	2-3
Cultures grown on 1X Vogel's salts and 2% sucrose for 3 days. Pad suspended on phosphate buffer containing 0.2 mg/ml DL-ethionine for an additional 48 hours, without shaking.	241	3
Cultures grown on 1X Vogel's salts and 2% sucrose for 3 days. Pad suspended on phosphate buffer containing 0.2 mg/ml D-phenylalanine for an additional 48 hours, without shaking.	241	2

the banding pattern reflects differences in the amount of tyrosinase present in the extract.

Changes in the Tyrosinase Electrophoretic Pattern as a Function of Time During Derepression

A convenient means for determining whether there is variation in the electrophoretic pattern of tyrosinase as a function of tyrosinase activity is to follow changes in the pattern during the course of derepression. For these studies, cultures of III 70 $\underline{T}^{PR}$ were derepressed by the Pall method, using either DL-ethionine or D-phenylalanine to initiate derepression. At 48 hours, extracts from such cultures displayed 3 bands of tyrosinase when derepressed with ethionine and 1 band of tyrosinase when derepressed with D-phenylalanine (refer to Table V). Cultures were harvested at various times after the addition of DL-ethionine or D-phenylalanine. The time course of derepression observed in two separate experiments is shown in Figure 2. Higher activities were obtained in cultures derepressed with D-phenylalanine than in DL-ethionine-treated cultures. A summary of the results obtained with paper strip electrophoresis of crude extracts prepared from pads harvested at each time point is given in Table VI. These results suggest that multiple forms of tyrosinase are found at early times during D-phenylalanine derepression, and at late times during DL-ethionine derepression. Conversely, a single electrophoretic form of tyrosinase is observed in D-phenylalanine-treated cultures after 12 hours of derepression, while DL-ethionine-treated cultures contain a single form

Figure 2. Time course of tyrosinase production in cultures derepressed by the addition of D-phenylalanine (○——○) or DL-ethionine (□——□). Cultures were grown at 25°C for 3 days on 1/2X Vogel's salts and 1/2% sucrose in 125 ml Erlenmeyer flasks containing 20 ml medium. Derepression was initiated by adding 2 mg D-phenylalanine or DL-ethionine to each flask. Incubation was continued for an additional 48 hours on a reciprocal shaker (80 strokes/min) at 25°C.

I and II refer to the results obtained in two separate experiments.

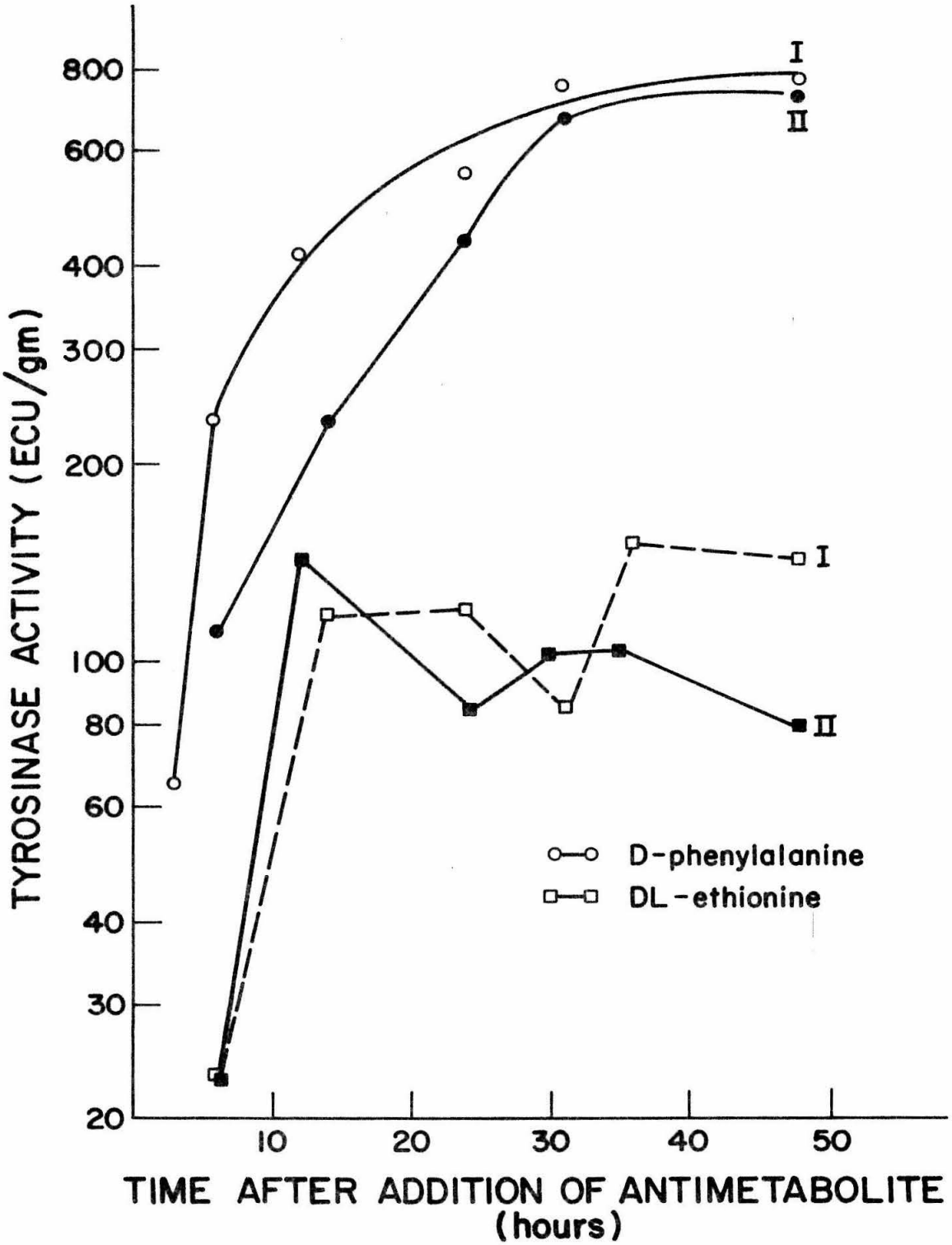

TYROSINASE ACTIVITY (ECU/gm)
800
600
400
300
200
100
80
60
40
30
20
10
20
30
40
50
TIME AFTER ADDITION OF ANTIMETABOLITE
(hours)
I
II
I
II
D-phenylalanine
DL-ethionine

TABLE VI

Changes in the tyrosinase electrophoretic pattern as a function of time during derepression by D-phenylalanine or DL-ethionine

Experiment I			Experiment II		
Hours	Tyrosinase ECU/gm	Bands	Hours	Tyrosinase ECU/gm	Bands
Derepression by D-phenylalanine:					
			3	65.1	2
6	111	2-3	6	235	3
			12	418	1
14	231	1			
24	438	1	24	558	1
			30	760	1
31	675	1			
48	735	1	48	780	1
Derepression by DL-ethionine:					
6	23.4	1	6	22.8	1
			12	143	2-3
14	118	3			
24	120	2	24	85	2
			30	103	2
31	85	2			
			35	104	2
36	152	3			
48	145	3	48	80	2

Experimental procedures as in Table I.

of tyrosinase at times prior to 12 hours of derepression. The explanation for these results is unclear, but they suggest that the banding pattern is influenced by changes in the cells which occur during derepression. No good correlation between total activity per gram wet weight of mycelium and the electrophoretic pattern is indicated by these results. The differences observed in cultures of III 70 $\underline{T}^{PR}$ derepressed for equal times in different experiments may explain the different patterns observed in sister spores during studies of the inheritance of multiple forms of tyrosinase.

Experiments Concerning the Nature of the Supernumerary Bands

The "extra" bands observed by Sueoka in T^{Sing-2} strains (1), and those observed during the present studies were unstable to brief heating (60°C for 2 minutes) in crude extracts. The experiments reported here attempted to determine whether the activity in the labile bands was destroyed by heating, or whether the bands were converted to material migrating to the "major" band position, as suggested by the studies of Fox and Burnett (3). The latter authors reported that the multiple electrophoretic forms of tyrosinase are stable after separation by continuous flow paper electrophoresis.

The 3 electrophoretically distinct forms of tyrosinase present in crude extracts of III 70 $\underline{T}^{PR}$ were separated by paper strip electrophoresis. The unsprayed strips were placed between two aluminum blocks which were preheated to 60°C and incubated for 15 minutes. A decrease in the intensity of all bands was observed compared to an unheated

control, but the supernumerary bands did not disappear. These results are consistent with the hypothesis that the supernumerary bands are converted to the "major" band upon heating of crude extracts, and that the isolated bands are not destroyed by heating at 60°C for 15 minutes under these conditions.

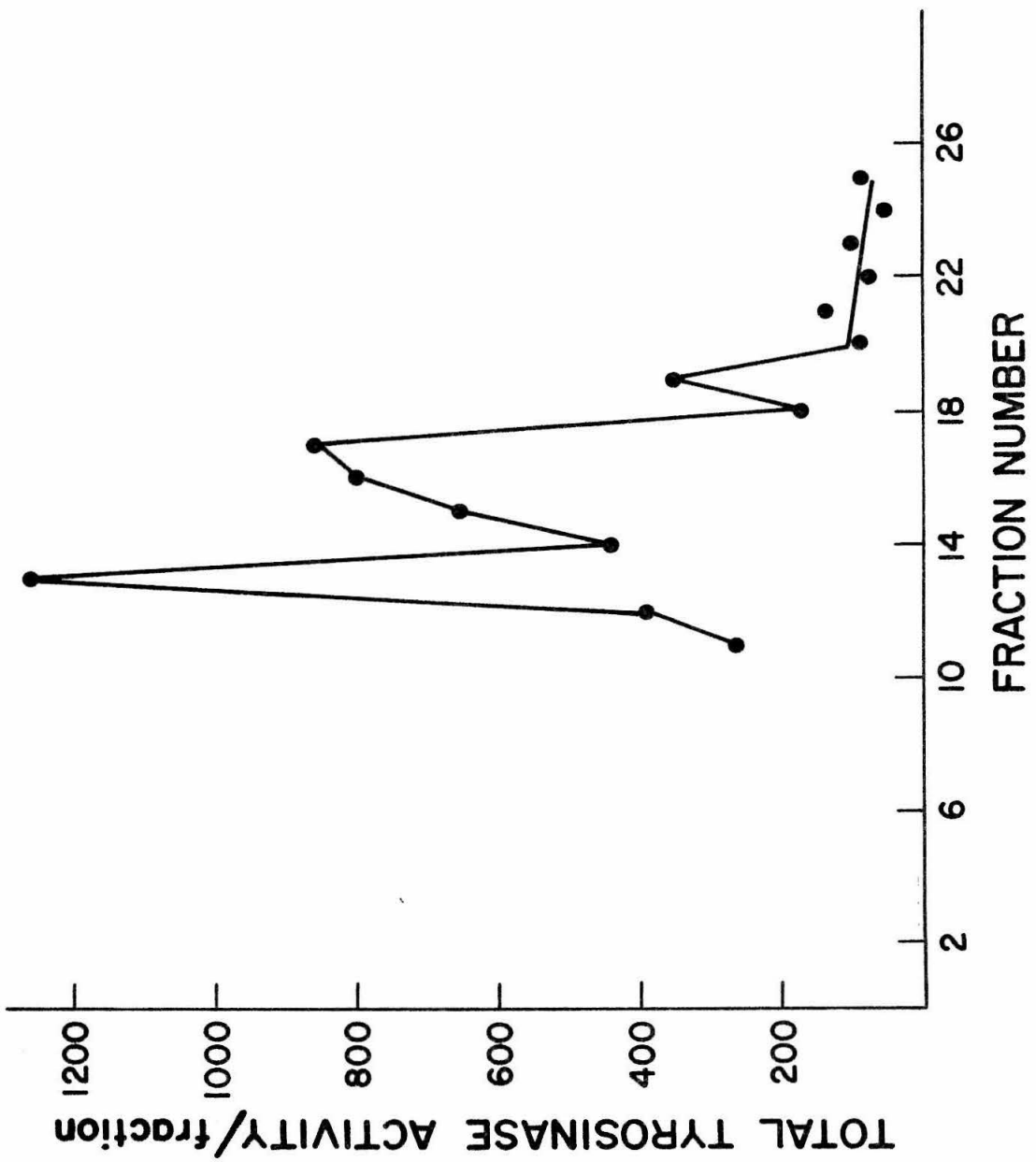
A second question concerning the nature of the supernumerary bands was whether they could be regenerated from the single band present in a heated extract. The "extra" bands were not regenerated by incubating the heated extract at 25°C for up to 2 hours, nor by adding crude extract from unheated repressed cultures. The postulated conversion of the "extra" bands to the "major" band appears to be irreversible.

Unsuccessful Attempts to Isolate the Supernumerary Bands

Continuous Flow Paper Electrophoresis

To better study the properties of the multiple forms of tyrosinase, homogeneous preparations of each form were desirable. Preliminary studies indicated that elution of pure fractions of each band from paper strips following electrophoresis was impractical. Continuous flow paper electrophoresis as described by Fox and Burnett (3), did not resolve the supernumerary bands of T^{PR} strains. Adding bovine serum albumin to the lower curtain to reduce adsorption of tyrosinase allowed separation of different electrophoretic forms, as shown in Figure 3, but fractions from the individual peaks all migrated to the same "major" band position in paper strip electrophoresis.

Figure 3. Elution profile of crude tyrosinase from 65-1434 $\underline{T}^{PR}$ fractionated by continuous flow paper electrophoresis. Cultures were grown on 20 ml 1/2X Vogel's salts and 1/2% sucrose for 3 days in 125 ml Erlenmeyer flasks at 25°C, without shaking. Derepression was begun by adding 2 mg D-phenylalanine per flask. Incubation was continued for 48 hours at 25°C on a reciprocal shaker (80 strokes/min). Electrophoresis was carried out in 0.02M sodium barbitol buffer, pH 8.6, containing 0.1 per cent bovine serum albumin. The lower curtain was wetted with 1 per cent serum albumin to reduce adsorption of tyrosinase to the paper. A 0.5 ml aliquot of each fraction collected at the bottom of the sheet was analyzed for tyrosinase activity. Data plotted are in arbitrary units (Δ Klett units/5'/fraction).


TOTAL TYROSINASE ACTIVITY/fraction
1200
1000
800
600
400
200
2
6
10
14
18
22
26
FRACTION NUMBER

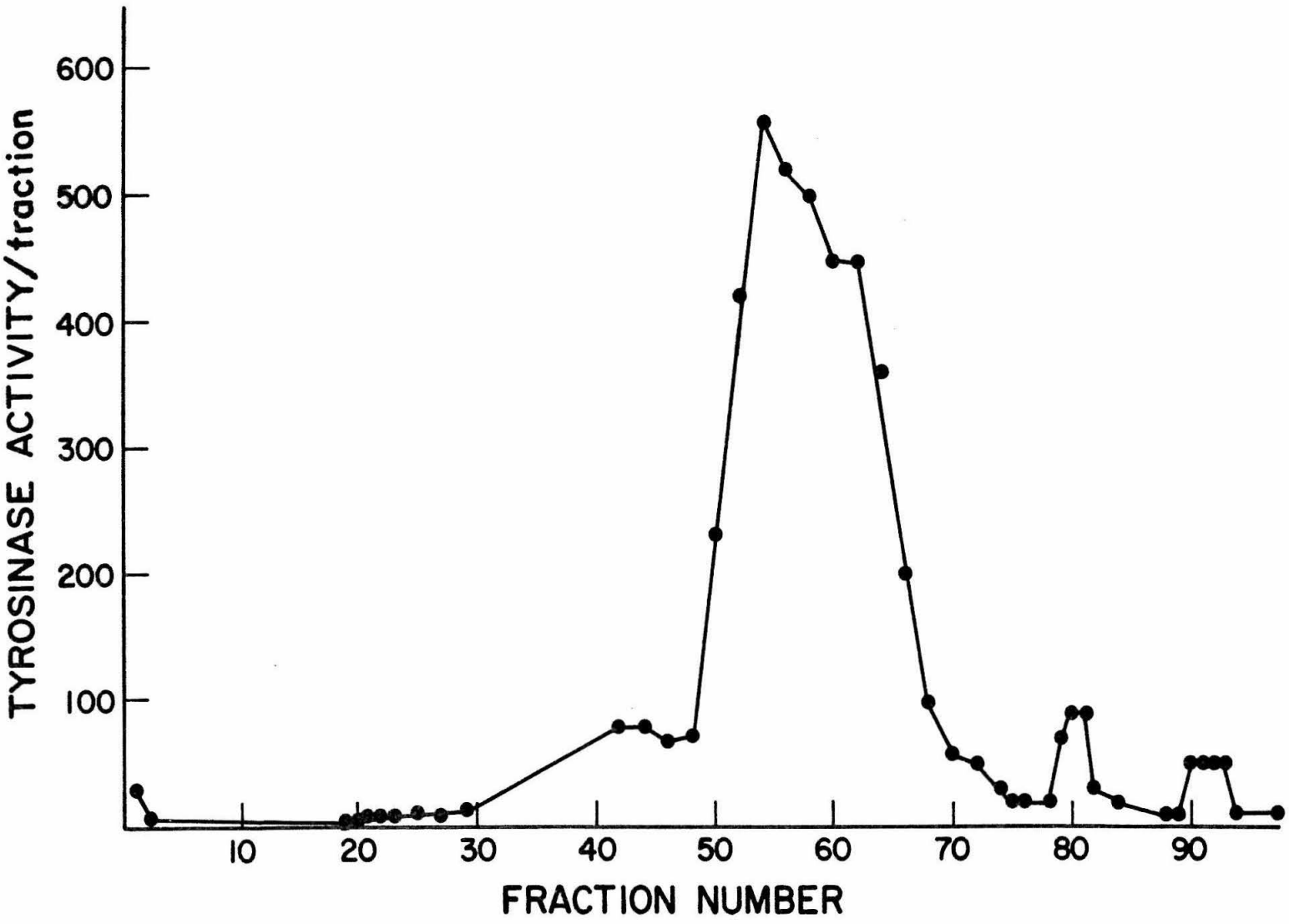
Zone Electrophoresis in a Density Gradient

The last technique tried to separate the individual tyrosinases of III 70 $\underline{T}^{PR}$ was zone electrophoresis in a sucrose density gradient. Electrophoresis of a crude extract under the conditions described in Materials and Methods appeared to resolve the tyrosinases as shown in Figure 4. Paper strip electrophoresis of the individual peaks indicated that all peaks migrate to the T_b^{PR} position. The crude extract run simultaneously contained all three peaks: T_a^{PR}, T_b^{PR}, and T_c^{PR}. Thus, the peaks isolated by preparative electrophoresis appear to convert to the "major" electrophoretic form.

To obtain a crude extract with a very high tyrosinase specific activity, and to determine whether the multiple forms of a T^L strain were also labile to preparative electrophoresis, strain XIX 12 $\underline{T}^L$ ($2\ T^L$) was used for zonal density gradient electrophoresis. The elution profile again showed asymmetry, suggesting the partial separation of different forms of tyrosinase. Paper strip electrophoresis was applied to samples taken from individual fractions throughout the region of tyrosinase activity. Tyrosinase from all fractions migrated to the T_b^L position. Apparently, the T_c^L tyrosinase fraction was converted to T_b^L during or after preparative electrophoresis. Recovery from the column was 62 per cent, allowing the possibility that T_c^L was lost by decay rather than by conversion.

In summary, all attempts to isolate the components observed after paper strip electrophoresis resulted in the loss of the supernumerary bands or their conversion to the "major" component. Further

Figure 4. Elution profile of crude III 70 $\underline{T}^{PR}$ tyrosinase fractionated by zone electrophoresis in a sucrose density gradient. Cultures were grown on 1/2X Vogel's salts and 1/2% sucrose for 3 days at 25°C in 125 ml Erlenmeyer flasks containing 20 ml medium. Cultures were derepressed by adding 2 mg DL-ethionine per flask. Incubation was continued at 25°C on a reciprocal shaker (80 strokes/min) for an additional 48 hours. Zone electrophoresis was carried out in 0.05M sodium phosphate buffer, pH 6, using a linear sucrose gradient (0-20% sucrose). A constant current of 30 ma was maintained for 19 hours at 2-4°C. One ml fractions were collected from the bottom of the column after completion of electrophoresis. A 0.1 ml aliquot of each fraction was assayed for tyrosinase activity. Data are plotted in arbitrary units (Δ Klett units/5'/fraction).


TYROSINASE ACTIVITY/fraction
600
500
400
300
200
100
10
20
30
40
50
60
70
80
90
FRACTION NUMBER

studies on the properties of the multiple electrophoretic forms of tyrosinase await their isolation in preparative quantities.

DISCUSSION

The results reported here indicate that multiple electrophoretic forms of tyrosinase can occur in Neurospora crassa homocaryons. That the multiple forms in each strain arise by modification of a single enzyme is suggested by several observations. The banding pattern observed after separation by paper strip electrophoresis of the different forms of tyrosinase present in a crude extract depends on the T allele present in the culture. That is, the locations of the supernumerary bands after electrophoresis of a T^{L} extract are not the same as the locations of the supernumerary bands from a T^{PR} strain. Results of crosses between T^{L} and T^{PR} strains are consistent with the notion that a certain group of sub-bands are associated with T^{PR} stocks, while another group of sub-bands are associated with T^{L} stocks. An analogous situation has been observed in studies of the alkaline phosphatases of E. coli (11). Single mutations in the alkaline phosphatase structural gene which change the charge of the protein affect all sub-bands identically, indicating that they are all products of the same gene (11). The nature and function of the sub-bands of alkaline phosphatase are unknown.

Of the 1 to 4 tyrosinase bands obtained from a crude extract from a Neurospora homocaryon, only one band is found after heating the extract at 60°C for 2 minutes; this treatment may convert the supernumerary bands to the "major" band. In support of this hypothesis is the observation that heating filter paper strips containing the separated bands of tyrosinase did not destroy the activity in the

supernumerary bands. A similar conversion of heterogeneous electrophoretic forms of an enzyme has been reported by Jacobson (12). In this case, alcohol dehydrogenase of Drosophila is converted from one form to another which differs in electrophoretic mobility and thermostability by binding nicotinamide adenine dinucleotide (NAD). The rate at which the conversion occurs in vitro is markedly increased by elevated temperatures. The multiple forms of alcohol dehydrogenase are found in nature and all map at the same site on the second chromosome. A single form of the enzyme is obtained by purification, just as the "major" band of T^{L}, T^{S}, or T^{PR} is the only band observed in purified tyrosinase preparations.

The tyrosinase electrophoretic pattern varies with the method of derepression used. Growth on low sulfur medium results in a crude extract which displays a single electrophoretic band of tyrosinase, which probably explains why multiple forms of tyrosinase were rarely observed during early studies by Horowitz and his associates. The Pall method of derepression (5) using DL-ethionine, results in the appearance of multiple forms of tyrosinase in most strains. Not only the method of derepression, but also the time during the course of derepression influences the electrophoretic pattern observed. The banding pattern appears to be altered by changes occurring within the cells during derepression. By analogy to the alcohol dehydrogenase of Drosophila, alterations in the banding pattern may reflect fluctuations in the concentration of some small molecule which binds to the enzyme and alters its electrophoretic mobility. Sub-banding of lactic dehydrogenase (LDH)

isozymes also appears to be influenced by the surrounding milieu. Whereas sub-bands can be created by dipping whole tissues in formaldehyde and storing the treated tissues at 3°C for several days, other tissues display sub-bands without formaldehyde treatment (13). The sub-bands appear to result from changes in the subunits of LDH which can occur before or during enzyme extraction. A resistance to alteration may be affected by the cellular environment present in certain tissues (13).

An interesting case of multiple electrophoretic forms of a protein which may be related to the sub-bands of tyrosinase concerns animal hemoglobins. Huisman et al. found that different patterns are observed after starch gel electrophoresis of hemoglobin, depending on the concentration of phosphate buffer used for dialysis of the total red blood cell hemolysate (14). If hemolysates were dialyzed against 0.01M phosphate buffer, three to four electrophoretic components were observed. Dialysis against phosphate buffers with molarities greater than 0.01M resulted in a loss of the fastest-migrating component. The explanation for these results is that hemoglobin binds phosphate, which alters the electrophoretic mobility of the protein (14). All extracts of Neurospora tyrosinase were prepared in 0.1M sodium phosphate buffer, and electrophoresis was carried out in phosphate buffer in most cases (an exception is continuous flow paper electrophoresis, which utilized sodium barbitol buffer). The behavior of tyrosinase sub-bands could be explained by a thermolabile attachment of phosphate to tyrosinase. Differences related to the time or method of tyrosinase derepression may

be explained by other factors which affect the binding of phosphate ion, such as the presence of competing ions in the extract.

Fox and Burnett (3) and Fuchs (4) report that the tyrosinase isozymes isolated by continuous flow paper electrophoresis are stable after separation from the crude extract. The sub-bands of LDH are also stable after isolation (13,15,16). No experiments were done during the present investigation which bear on this point, because the sub-bands present in paper strip electrophoresis were not recovered from preparative electrophoresis. The sub-bands may have been converted to the "major" band during or after preparative electrophoresis, or may have been denatured without conversion to the "major" band.

Although the studies reported here do not elucidate the source of multiple banding in crude extracts of Neurospora, they are consistent with the hypothesis that the sub-bands arise by alterations of a single protein whose primary structure is specified by the tyrosinase structural gene (T).

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