GENETIC AND BIOCHEMICAL STUDIES OF TYROSINASE IN NEUROSPORA

AND

LACCASE IN NEUROSPORA

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ABSTRACT

Part I

Genetic and Biochemical Studies of Tyrosinase in Neurospora

1) A new form of tyrosinase of Neurospora crassa (Sing-2) was found, which has different electrophoretic behavior and thermostability from the three previously known forms (T^{L}, T^{S}, and T^{PR15}).

2) The characteristics of the new form are determined by the single locus, T, which also controls the characteristics of the other forms.

3) The kinetics of thermal inactivation of the different tyrosinases were studied in detail at different temperatures.

4) Two tyrosinaseless genes (ty-1 and ty-2) are independent from each other and from the T-locus, and both of them are epistatic to the T-locus.

5) Heterocaryons of the following genotypes were produced.

Het.A $(T^{S},hist,ty\text{-}1)(T^{S},ad,ty\text{-}1^{+})$
Het.B $(T^{L},hist,ty\text{-}1)(T^{S},ad,ty\text{-}1^{+})$
Het.D $(T^{L},hist,ty\text{-}1)(T^{Sing2},ad,ty\text{-}1^{+})$

It was found that a) the ty-1 allele is recessive to its normal form, $ty\text{-}1^{+}$, b) Het.B and Het.D produce a mixture of both forms of tyrosinase determined by their genotypes, and c) the ratio of the two enzyme forms produced corresponds to the ratio of the two component nuclei in the heterocaryons.

6) The significance of the present findings for the gene-enzyme relationship is discussed.

Part II

Laccase in Neurospora

The "second phenol oxidase" in Neurospora reported by Horowitz and Fling (1953) was further studied.

1) The enzyme was purified, characterized as to substrate specificity, inhibitor spectrum, and pH optimum, and identified as a laccase.

2) Production of laccase shows wide variability among different strains of Neurospora and is influenced greatly by external factors, such as temperature, concentrations of sulfur and copper of the medium.

3) Immunological studies show that there is no serological similarity between laccase and tyrosinase of Neurospora.

4) Inducibility of laccase in Neurospora is a variable character, but seems to be strain specific.

TABLE OF CONTENTS

PART I

I. INTRODUCTION

The main problem in biochemical genetics concerns the primary function of the gene in the synthesis of an enzyme. Evidence that there is a correlation between changes in genes and enzymes (and other proteins) is overwhelming (1, 2). There are two aspects of evidence in this basic problem. One has to do with quantitative changes in enzyme activity induced by gene mutations--e.g., a gene mutation may lead to the absence of an enzyme (1) or may restore an enzyme abolished by a previous mutation (suppressors) (3,4). The other aspect has to do with qualitative changes in the structure of the molecule caused by gene mutation (2).

The instances where these two aspects of gene function can be analyzed in a single enzyme (or protein) system are, however, not many at present. Human hemoglobin is one of the few cases (5,6) but there are some intrinsic difficulties in the genetic analysis of the system.

Tyrosinase in Neurospora crassa is another case. In 1953 Horowitz and Fling (7) reported two qualitatively different forms of tyrosinase, thermostable (T^S) and thermolabile (T^L), and showed that the difference is determined by two alleles of a single locus T. The two forms have also been shown to differ in electrophoretic behavior (8). Heterocaryons carrying the two alleles in a common cytoplasm produce a mixture of the two corresponding forms of tyrosinase (9). These facts are consistent with the idea

that one gene is responsible for the structure of this enzyme (1,7,10).

Two more forms of the enzyme have been discovered, and genetic and biochemical analysis of one of them (T^{Sing2}) will be described in this thesis.

Quantitative variations in tyrosinase content have attracted our attention following the finding of tyrosinase-less strains by Dr. M Westergaard, University of Copenhagen (11). Several such strains have been sent to our laboratory and two of them have been analyzed. An important part of present research involves the analysis of the genetic behavior of these tyrosinaseless characters in relation to the T-alleles. The results clarified some aspects of gene action on enzyme formation.

II. STRAINS AND METHODS

The strains

Strains, 4-121A and 4-137a, have been used. These strains were isolated by Horowitz and Fling (7) as the producers of the thermostable tyrosinase (T^S-form) and the thermolabile tyrosinase (T^L-form), respectively. A new form of tyrosinase, called Singapore-2, has been found in a culture sent from Singapore. Another form, PR-15, originating in a strain sent by Dr. Howard Teas from Puerto Rico has been characterized also in our laboratory (12).

The biochemical markers used in heterocaryons are reisolates of histidineless (C-140 or hist-3), adenineless (35203 or ad-3) and their derivatives. Tyrosinaseless strains, ty-1 and ty-2, are mutants of natural origin and have been sent by Dr. M. Westergaard. The ty-1 has a morphological characteristic, "velvet or vt," along with the absence of tyrosinase production and the two characters have not been separable by crossing over so far. The strains which have been isolated by Dr. Westergaard are designated by putting "W" before the strain number and those isolated by the author by "NS." Both tyrosinaseless strains are female-sterile (11,13).

Culture of Neurospora

The stocks of wild-type strains are maintained on complete agar slants and biochemical mutants on Fries minimal medium supplemented with the required compounds. For

tyrosinase production cultures were usually grown for four days at 25°C on the low sulfur liquid medium described by Horowitz and Shen (14), 20 ml of which was dispensed into each 125 ml Erlenmyer flask. The histidineless and adenineless strains were supplemented with 2 mg of histidine hydrochloride and 1 mg of adenine hydrochloride respectively. Production of tyrosinase by adenineless mutants takes a longer period of incubation (six days) and supplementation with 2 mg adenine HCl has recently been found to give higher production.

Extraction and purification of tyrosinase

Crude extracts of tyrosinase were prepared from mycelium ground in a mortar with sand and 2 ml of 0.1M sodium phosphate buffer, pH 6.0, per gram wet weight of mycelium, centrifuging off the sand and debris. A partial purification was carried out by the method of Horowitz and Fling (7). Ten- to fifty-fold purification was obtained depending on the initial specific activity of the crude extract. The following convenient way to concentrate the enzyme preparation was used. The crude extract is put in a dialysis bag and immersed in cold 80 per cent saturated ammonium sulfate solution. The "dialysis" was carried out in the refrigerator for five to twelve hours with occasional stirring. The volume of the preparation reduces to one fourth to one fifth of the original volume, and all tyrosinase activity is in the thick precipitate, which is centrifuged, dissolved in

the desired volume of buffer, and dialyzed against buffer overnight, changing buffer once.

The assay of tyrosinase activity

Tyrosinase activity was determined colorimetrically with 3,4-dihydroxy-DL-phenylalanine (DL-DOPA) as the substrate by the Klett-Summerson colorimeter with No. 42 filter (blue) (14). Sodium phosphate buffer of 0.1M pH 6.0, was used throughout the experiments. One ml of 0.02M DL-DOPA is added to 4 ml of the sample for assay which has been equilibrated at 30°C for at least five minutes. The red product of the reaction (dopachrome) was measured. An enzyme unit is defined as the amount of enzyme that produces a reading of one colorimeter unit in five minutes under the above conditions (colorimeter units or c.u.).

Heat treatment

Most of the heat-inactivation experiments were carried out at 59°C. The fluctuations in the bath temperature were quite small (± 0.02). Two different procedures have been used (7). In the first, 0.5 ml aliquots of the enzyme solution were measured into a series of Klett-tubes held in a rack. The tubes were removed at intervals and rapidly chilled in ice. The samples were diluted to 4 ml with buffer before assay. In the second procedure a single 50-ml tube containing a relatively large volume of buffer was brought to temperature equilibrium in the water bath, for which five minutes were quite enough from the room tempera-

ture to 59°C. A small volume of enzyme solution was added to the tube at zero time. Samples of 4 ml were removed at intervals and chilled. The second method is referred to as the "dilution method."

Electrophoresis

The Spinco apparatus of hanging strip paper electrophoresis (Durrum type) was used at 10°C with 0.05M sodium phosphate buffer, pH 6.0. The procedure which will be described has been developed by Horowitz and Fling (8). First, eight paper strips were dipped in 100 ml of 1% bovine serum albumin (BSA) solution in buffer and set on the rack. The remaining BSA solution was combined with 900 ml of buffer and used in the terminal reservoirs. The amount of tyrosinase applied onto a strip is 10-30 c.u., which corresponds to 0.01 - 0.06 ml of enzyme preparation. Ten milliamperes of electric current were used for about 16 hours. After electrophoresis the strips were sprayed with DL-DOPA solution, 0.02M in 0.1M sodium phosphate buffer, pH 6.0. The location of the enzyme is easily detected by a rapid development of red color on the strip. The color band is usually narrow (ca 5 mm) and sharp.

III. A NEW FORM OF TYROSINASE FROM SINGAPORE-2 STRAIN

Comparisons and characteristics

One of the strains sent from Singapore, Singapore-2 strain (Sing-2), produces tyrosinase with different characteristics from those of other standard types. The thermostability, energy of heat inactivation and electrophoretic mobility of the tyrosinase from the Sing-2 strain and other strains are summarized in Table 1. The electrophoretic mobility of Sing-2 tyrosinase is quite reproducible and gives a uniform value among derived strains. The heat stability, however, varies considerably at 59°C from strain to strain and even from preparation to preparation from the same strain with or without purification. A similar variability has been found also in tyrosinase of a few T^S derivatives (15). (It should be noted, however, that the variability is not so large as to cause difficulty in distinguishing between the different forms of the enzyme in crosses, except $T^{Sing2} \times T^S$). Thermostabilities of T^{Sing2}-, T^S-, and T^L-tyrosinase were, therefore, analyzed further at different temperatures. The dilution method was used and at least four time-points were taken for each temperature. Kinetics of the inactivation for each case was of the first order (Fig. 1). The Arrhenius plots of the stability of various preparations are shown in Figs. 2, 3, and 4, based on the equation:

Table 1. Properties of tyrosinase of various strains of Neurospora crassa.

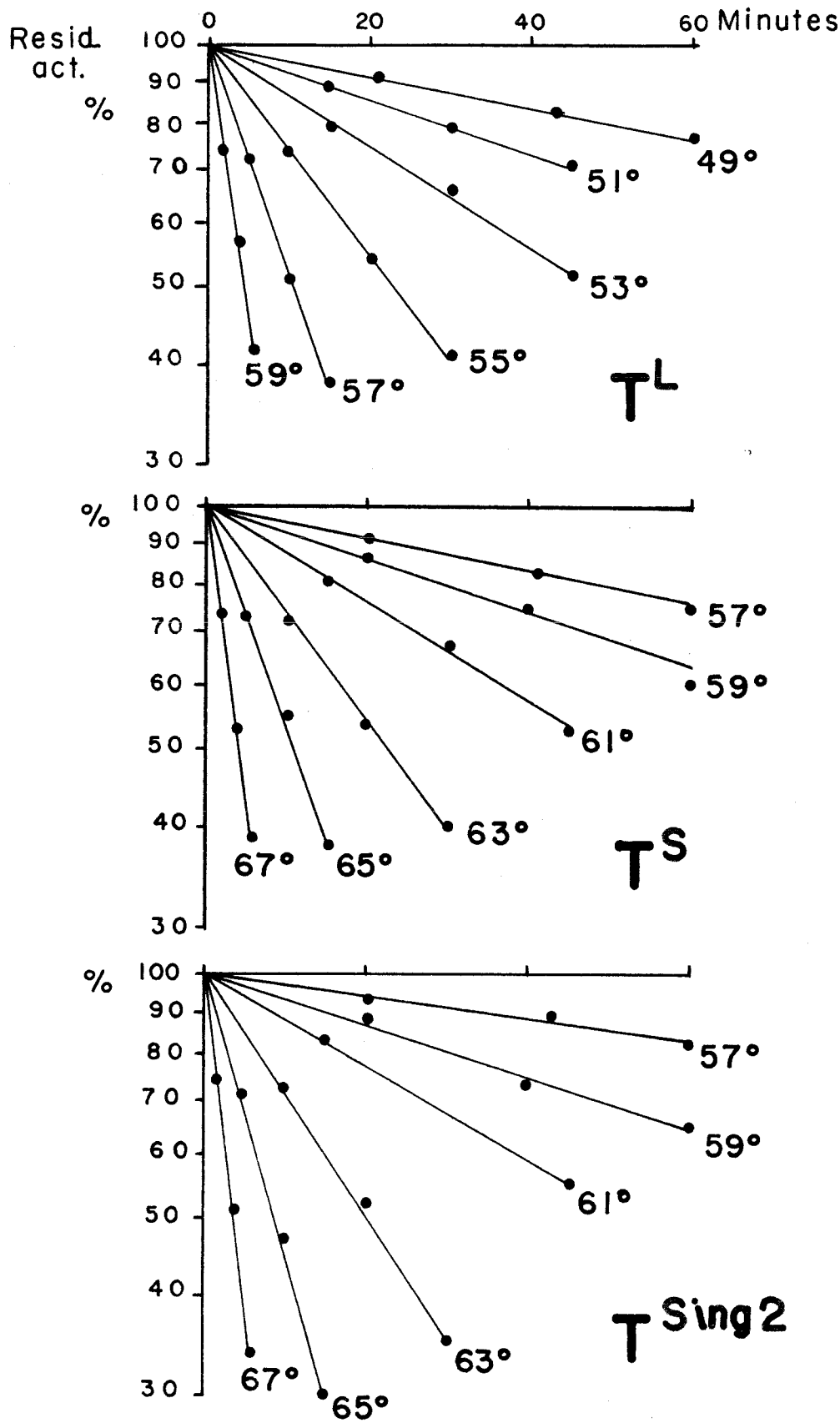
	Sing-2 (T^{Sing2})	4-121 (T^{S})	4-137 (T^{L})	Puerto Rico-15 (T^{PR15})
Thermostability				
$t_{1/2}$ at 59°C	70',[1]	70',[1]	5',[2]	20'
$t_{1/2}$ at 62°C	35'	35'	0.5'	-
Break points	61°C	61°C	53°C	-
Energy of activation (cal per mole)				
μ above break pt.	96,000	90,000	84,000	-
μ below break pt.	ca 36,000	ca 61,000	59,000	-
Entropy of activation (entropy unit)				
ΔS above break pt.	+207	+189	+178	-
ΔS below break pt.	ca +25	ca +102	+101	-
Electrophoretic[3] migration on paper (mm/hr)	1.5	2.0	2.25	1.5

1) These values represent majority cases. For variations, see Figs. 2 and 3.

2) Half-life of 3 min has been reported for Na-K-phosphate buffer (0.1 M, pH 6.0). In present experiment Na-phosphate buffer (0.1 M, pH 6.0) was used exclusively.

3) Na-phosphate buffer, 0.05 M, pH 6.0, 10 mA, at 10°C.

Figure 1: Thermal inactivation of T^{L}-, T^{S}- and T^{Sing2}-forms of Neurospora tyrosinase.



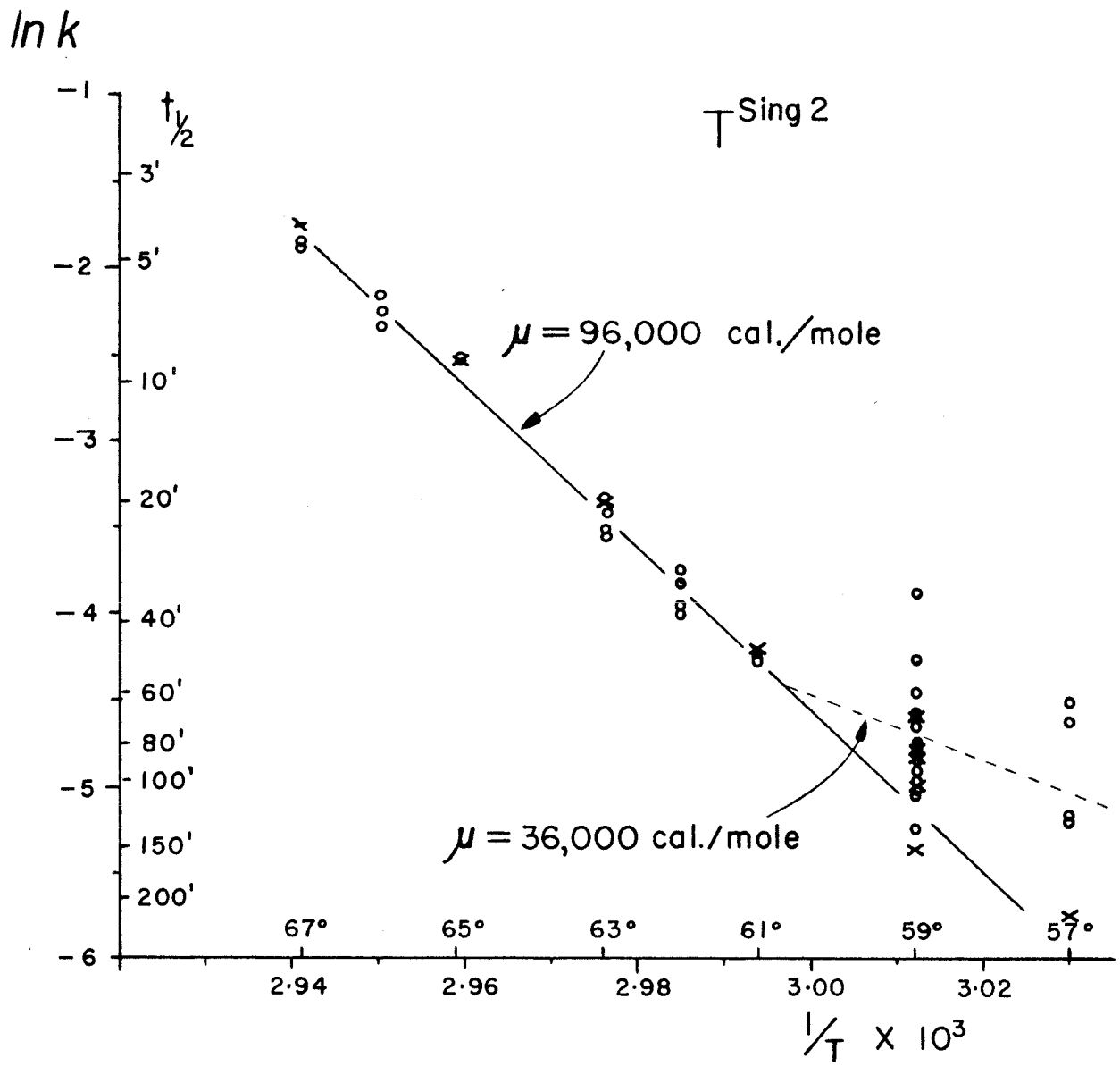


Figure 2: Arrhenius plots for inactivation of T^{Sing2}-tyrosinase. Circles represent purified preparations, and crosses crude preparations.

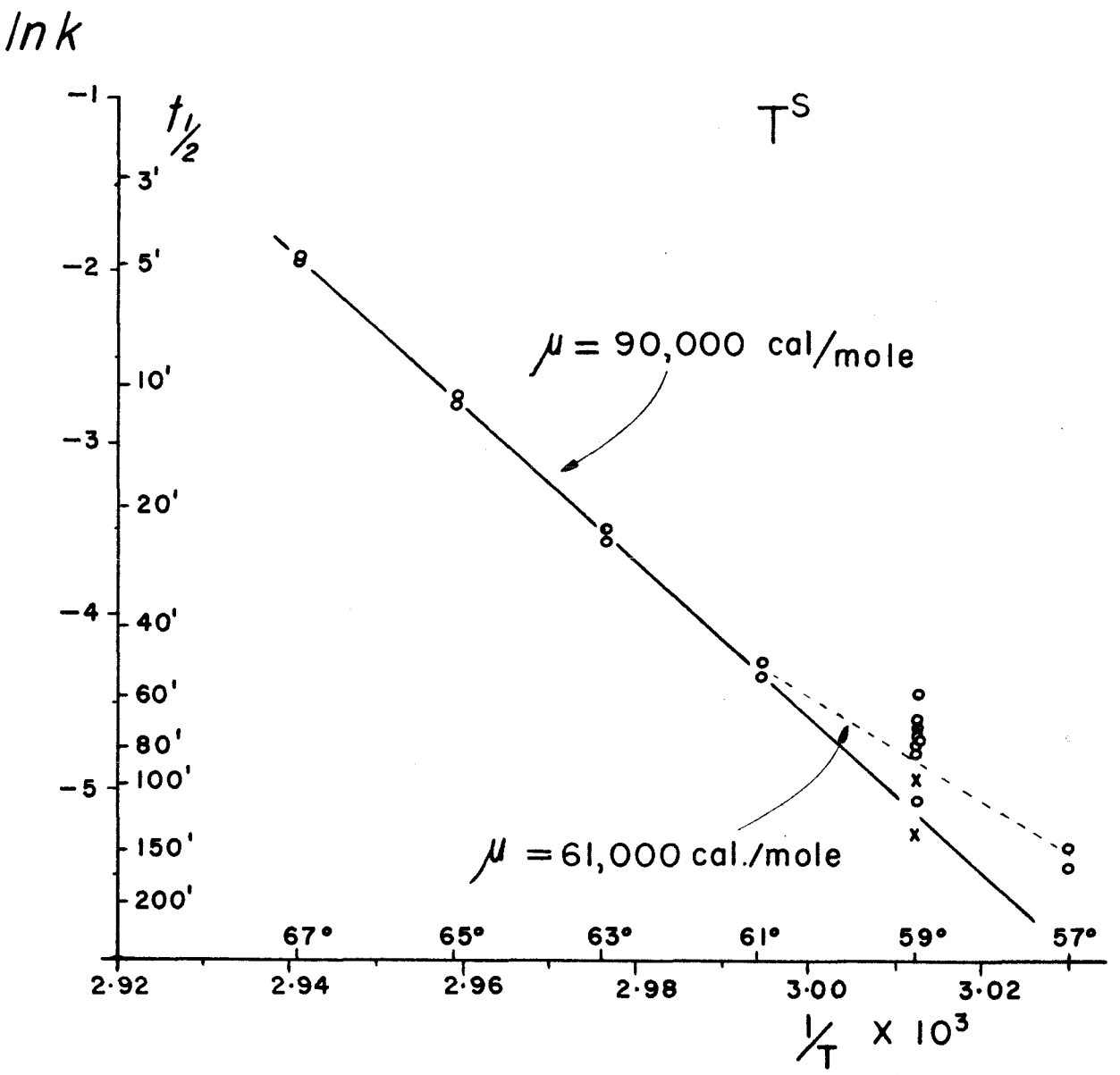


Figure 3: Arrhenius plots for inactivation of T^S-tyrosinase. Circles represent purified preparations, and crosses crude preparations.

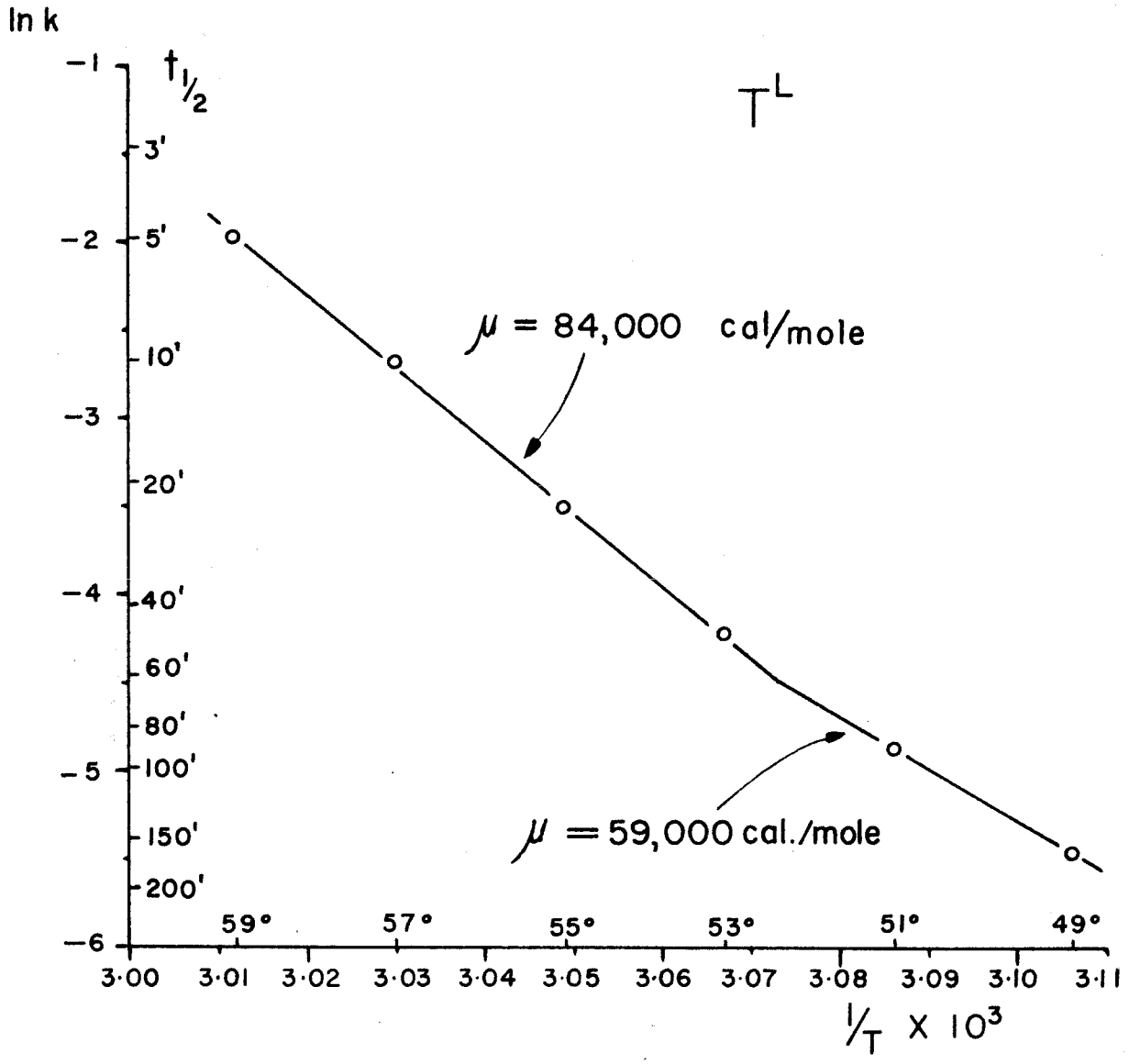


Figure 4: Arrhenius plots for inactivation of T^L-tyrosinase. Circles represent purified preparations, and crosses crude preparations.

$$\ln k = \frac{A}{R}\left(\frac{1}{T}\right) + C \qquad (1)$$

where k is the inactivation rate constant (per min), R is the gas constant (1.987 cal per degree per mole), T is the absolute temperature, A (or μ) is the activation energy (cal per mole), and C is a constant.

According to the above equation, a straight line should be obtained when lnk is plotted against 1/T.

The half-life ($t_{1/2}$) of the enzyme activity was measured from the inactivation curve on a semi-log graph. The inactivation rate constant, k, can be calculated from equation 2,

$$k = \frac{\ln 2}{t_{1/2}} = \frac{0.69}{t_{1/2}} \qquad (2)$$

and lnk from equation 3.

$$\ln k = \ln(\ln 2) - \ln t_{1/2} = -(0.367 + \ln t_{1/2}) \qquad (3)$$

The activation energy of tyrosinase inactivation, μ, was calculated from the Arrhenius equation (3).

$$\ln \frac{k_2}{k_1} = \frac{A}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) \qquad (4)$$

where k_1 and k_2 are rate constants at T_1 and T_2, respectively.

The entropy of the activation for the heat inactivation was calculated by equation 5 (16) following Sizer's description (17).

$$k = k' \frac{KT}{h} e^{-E/RT} e^{\Delta S/R} \qquad (5)$$

k: the rate constant of inactivation.
k': the transmission coefficient which can usually be assumed to be unity.
K: Boltzmann's constant.
E: the activation energy which in enzyme reactions is essentially equal to μ.
ΔS: the entropy of activation.

The following points are evident from the results: 1) both Sing-2- and T^S- forms of Neurospora tyrosinase give essentially similar heat inactivation kinetics, 2) above 61°C the half-lives of Sing-2- and T^S-tyrosinase preparations from different strains show less variation than below 61°C, 3) in the range of 61°-67°C the plots follow Arrhenius kinetics, while below 61°C the slope deviates towards the labile direction from that drawn for the data above 61°C. Arrhenius plots of a purified preparation of 4-137 (T^L) show a break at about 52°C. These discontinuities may be interpreted as meaning either that in the lower temperature range some heat-labile non-dialyzable materials make the tyrosinase molecule more labile than its intrinsic value ($T_{1/2}$ = 120 min at 59°C for Sing-2- and T^S-forms, obtained from extrapolation from the higher temperature range), or that the Arrhenius plots of all forms have discontinuities, above and below the break points they have different energies and entropies of activation (Figs. 2, 3, and 4). In the latter possibility the wider variation of the stability at lower temperatures for some Sing-2- and

T^S-forms may also be interpreted as indicating the presence of impurities. Horowitz and Fling (9) have previously noticed the wider variation in thermostability in T^S-form than in T^L-form and explained it on the following basis:

> The effective impurity seems to be principally tyrosine, traces of which are apparently released by a proteolytic enzyme which contaminates some of the tyrosinase preparations. Like other tyrosinases, Neurospora tyrosinase is inactivated in reacting with its substrates, and the presence of tyrosine in the preparations causes an apparent decrease in the thermostability of the stable form; the labile form is hardly affected, since it is thermally inactivated too rapidly at 59°C to engage in many catalytic cycles.

This is also a probable explanation for the present case. The possibility of heterogeneity in tyrosinase itself seems to be excluded by the monomolecular kinetics of the inactivation at each temperature. If a new line is drawn in the range of 57°-59°C for Sing-2- and T^S-forms (through the average values of lnk's) and 49°-51°C for T^L-form, different values of activation energies and entropies of thermal inactivation are obtained from those derived at higher temperatures (Table 1). Among T^L-strains the variation in tyrosinase stability has been quite small at 59°C. Data for lower temperatures are, however, not available.

Genetics

It is known that T^S and T^L are alleles and the evidence that T^{PR15} is also an allele of the same locus has recently been obtained in our laboratory (12). To test the possible allelism of T^{Sing2} with the other forms, the following three

crosses were planned: $T^{Sing2} \times T^{S}$, $T^{Sing2} \times T^{L}$, and $T^{PR15} \times T^{Sing2}$. The Singapore strain was rather infertile with the other. However, two generations of backcrosses were carried out to improve the fertility. The backcross program is shown in Fig. 5. Random spores from the backcrosses were separately cultured to test the electrophoretic behavior and only strains with Sing-2 migration were chosen for further crosses.

From the cross, $T^{Sing2} \times T^{S}$, 23 complete asci were dissected into four pairs of ascospores in serial order, and each spore pair was cultured and the tyrosinase from it was investigated for electrophoretic migration. All of the asci showed a 1:1 segregation for Sing-2 and T^{S} migrations. Segregation for thermostability was not studied, since the two forms are practically identical in this respect.

The cross, $T^{Sing2} \times T^{L}$, was analyzed in 30 complete asci both electrophoretically and by heat-inactivation. Differences in electrophoretic migration of the two forms, T^{Sing2} and T^{L}, is so large (ca 11 mm after 17 hours), that the segregations were very clear (Fig. 6). The heat stability was conveniently tested by the following procedure. A crude extract was prepared from each strain. The enzyme activity was measured, and aliquots containing about 100 cu. were pipetted into two tubes. Buffer was added to each tube to make the volume 0.5 ml. One of the two tubes was not heated and the other was heated for ten minutes at 59°C, and the

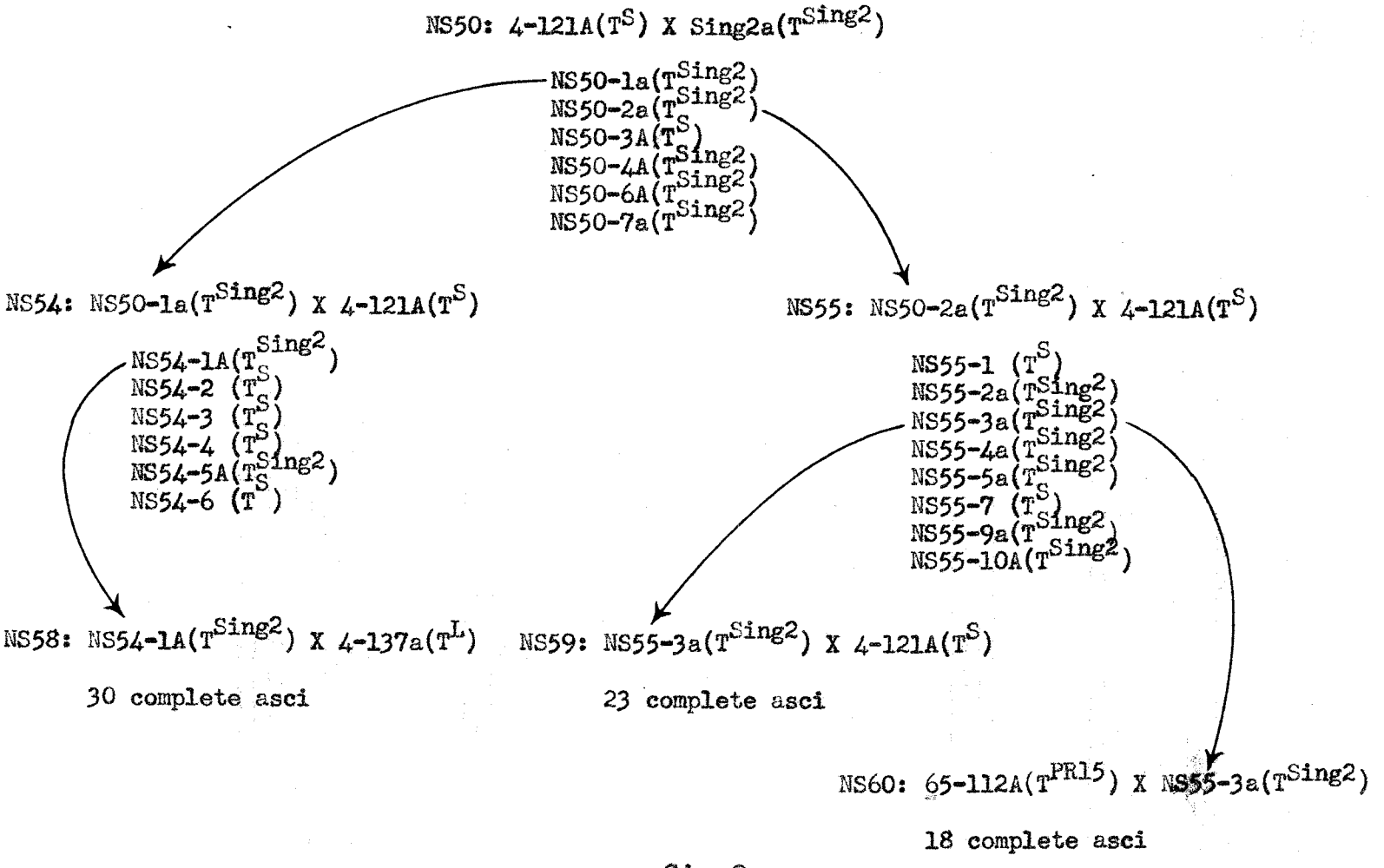
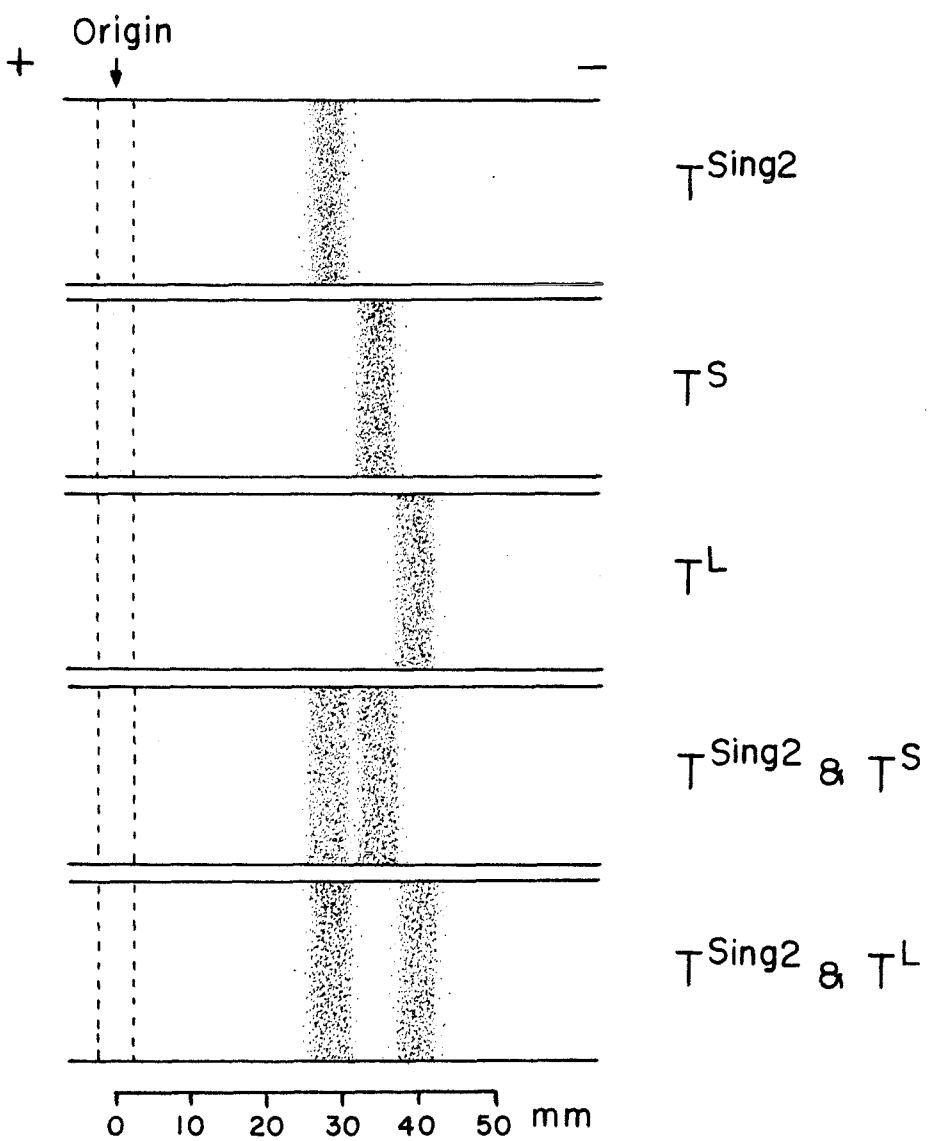


Figure 5. Pedigrees of T^{Sing2} strains used in various crosses.

Figure 6. Electrophoretic behavior of T^{Sing2}, T^{S}, and T^{L} tyrosinases and some mixtures. (0.05 M Na-phosphate-buffer, 1.25 mA per strip for 16 hours).



activity remaining was measured. The frequency distribution of the ratios of activity before and after heating are shown in Table 2. The distribution of the activity ratios separates into two groups with no overlap, and coincides with the electrophoretic behavior. Within each ascus, segregations for heat stability were obvious. These results clearly show that the two characters--thermostability and rate of electrophoretic migration--segregate 1:1, and no recombination has been observed between them. This agrees with the idea that both characteristics are determined by the T-locus, although the possibility of pseudoallelism cannot be excluded.

During the analysis of electrophoretic migrations of the segregants of the T^{Sing2} x T^{L} cross a peculiar phenomenon has been found. Fresh crude extracts from seven out of 60 analyzed Sing-2 type strains showed more than one band of tyrosinase activity; namely, three strains gave two bands and four strains three bands. One of those bands had a typical Sing-2 migration. Various experiments have been made on this phenomenon, and it has been found that this multiple band phenomenon is not reproducible among different preparations from the same strain and the components with migrations different from that of Sing-2 tyrosinase are extremely labile. Dialysis, freezing and thawing, keeping in refrigerator overnight, purification, brief heat treatment, etc. lead to the disappearance of the extra bands.

The cross between T^{Sing2} and T^{PR15} was analyzed in

Table 2. Distribution of ratios of the tyrosinase activity without heating and the activity after heating for 10 minutes at 59°C for the progeny strains of T^{Sing2} X T^{L} cross.

Activity ratio $A_{10'}/A_{0'}$ [1]	No. of strains	Electrophoretic behavior	Statistics: Total no. of strains	Statistics: Ave. ratio	Statistics: Ave. $t_{1/2}$
0.05	12	T^{L}	60	0.153 ±0.0087	3.7'
0.15	35				
0.25	12				
0.35	1				
0.45	2	T^{S}	60	0.823 ±0.021	38'
0.55	2				
0.65	11				
0.75	12				
0.85	11				
0.95	14				
1.05	6				
1.15	2				

1) A ratio higher than unity is probably due to heat-activation of protyrosinase in crude extracts (14,18).

18 complete asci and heat stability was tested. Again the segregation was 1:1. Segregation for electrophoretic migration was not studied, for the two forms have a practically identical rate of migration. These results are summarized in Table 3.

The genetic facts favor the possibility that the four different forms of tyrosinase are determined by four alleles at the single locus T. The chance that the present results (no recombination between Sing-2 and the other three forms of the enzyme) would have been obtained with non-allelic genes can be calculated by the use of Stevens' table (19). The results of the calculations are shown in the last row of Table 3 , where the upper limits of the possible recombination frequencies are given for 95% confidence limits.

Table 3. Summary of genetic analysis of Sing-2 tyrosinase.

	$T^{Sing2} \times T^{S}$	$T^{Sing2} \times T^{L}$	$T^{PR15} \times T^{Sing2}$
No. of complete asci analyzed	23	30	18
1st division segregation	6	8	4
2nd division segregation	17	22	14
Percentage of 2nd division segregation	74.0	73.4	77.8
Upper limits of[1] map distance possible at 5% level	6.1 cM	4.8	7.7

1) See the text.

IV. TYROSINASELESS STRAINS

Two strains, tyrosinaseless-1 and tyrosinaseless-2, of natural origin which produce little or no tyrosinase were sent by Dr. M. Westergaard to our laboratory. Strain ty-1 has a morphological characteristic ("velvet") on agar medium. This mutant morphology has not been separable genetically from the absence of tyrosinase. Both ty-1 and ty-2 behave as single gene mutants and both are female sterile, so that they can be used only as conidial parents. The original strain of ty-1, W913-83, has not produced any detectable tyrosinase in various culture conditions within the sensitivity of assay, with which one can detect less than 0.1% of the activity of standard strains.

A. ty-1

Dr. M. Fling of our laboratory has made some genetic studies of ty-1 by crossing it with the T^L-strain. The results are shown in Fig. 7. Her experiments show clearly, first, that the ty-1 locus is different from the T-locus and freely recombines with it; second, the blocking of tyrosinase production by ty-1 is not specific for a particular form of tyrosinase, T^S or T^L; and, third, some ty-1 derivatives produce small amounts of tyrosinase, which indicates the incompleteness of the blocking and its variability in expression due probably to other genetic factors. The following questions may be asked about the mode of interactions

Figure 7. Genetic analysis of ty-1 (M. Fling, personal communication)[1]

31: W913-83A($T^{(S)}$,ty-1) X 4-147a(T^{L},ty-1^{+})

ascus-1		ascus-2		ascus-3	
31-181(T^{S},ty-1^{+})	127[2]	31-221(T^{S},ty-1^{+})	44	31-521(T^{S},ty-1^{+})	172
31-182(T^{S},ty-1^{+})	77	31-222($T^{(S)}$,ty-1)	0	31-522($T^{(L)}$,ty-1)	0
31-183($T^{(L)}$,ty-1)	0	31-223(T^{L},ty-1^{+})	44	31-523($T^{(S)}$,ty-1)	0
31-184($T^{(L)}$,ty-1)	1	31-224($T^{(L)}$,ty-1)	0	31-524(T^{L},ty-1^{+})	157

41: 31-222A($T^{(S)}$,ty-1) X 4-137a(T^{L},ty-1^{+})

41-111(T^{L},ty-1^{+})	147
41-112($T^{?}$,ty-1)	6
41-113($T^{?}$,ty-1)	4
41-114(T^{S},ty-1^{+})	120

40: 31-523A($T^{(S)}$,ty-1) X 4-137a(T^{L},ty-1^{+})

40-111($T^{?}$,ty-1)	1
40-112(T^{S},ty-1^{+})	115
40-113($T^{?}$,ty-1)	11
40-114(T^{L},ty-1^{+})	120

1) The forms of tyrosinase which cannot be determined directly in ty-1 strains and are therefore deduced from the pedigree are shown in parenthesis, e.g. $T^{(S)}$ and $T^{(L)}$.

2) Relative production of tyrosinase activities.

of the T- and ty-1 loci concerning the synthesis of tyrosinase: 1) Is the blocking of tyrosinase synthesis by ty-1 an intranuclear phenomenon? 2) Does the ty-1 allele control the production of an inhibitor of tyrosinase or is it related to the inability to produce essential factors for the synthesis of tyrosinase? 3) Is the ty-1 allele a dominant character? 4) Are any other enzymes also missing in ty-1 strains? These questions are equally applicable to the other tyrosinaseless gene, ty-2.

The first and third questions are genetic ones which nevertheless cannot be tested by crossing a haploid organism like Neurospora, but heterocaryons of the type (m_1,m_2^+,T^S,ty-1) (m_1^+,m_2, T^L,ty-1^+) may give some clue to these questions, where m_1 and m_2 are biochemical markers. This type of experiment will be described in detail later. The second question has been tested by mixing experiments. The addition of an extract from the ty-1 strain to a preparation of tyrosinase did not show any indication of the presence of inhibitors in the ty-1 extract (15). Although there remains the possibility that inhibition occurs in the process of enzyme synthesis and not to the enzyme itself, the simple inhibitor hypothesis is excluded. The fourth question is an interesting one because if, for example, ty-1 has something to do with the production of prosthetic group of the enzyme, then some other enzymes having the same component in their molecule may be also missing in the same strain. It is known

that tyrosinase has copper in it as a functional site, so that other copper-containing enzymes might also be missing in ty-1 strains. The original strain, W913-83, in fact, lacks the "second phenol oxidase" (7) which is now identified as the copper-containing enzyme, laccase (20,21). Most of ty-1 strains are deficient in laccase, but some of them produce it up to the level of good producing strains. This indicates that the simultaneous deficiency of the two enzymes in W913-83 is fortuitous. Incidentally, among 17 wild strains tested for the production of laccase, seven of them produced little. Another possible copper-containing enzyme, uricase, is present in W913-83 (22). The copper uptake from the medium has been tested for a ty-1 strain and the wild type 4-121. It is found that the ty-1 strain takes up copper from the medium as rapidly as the wild strain. These are negative results and do not exclude the possibility that ty-1 may have some function in blocking the incorporation of copper atoms into the tyrosinase molecule, although the hypothesis in its simplest sense seems to be unlikely.

B. ty-2

The genetic analysis of ty-2 was initiated by H. Macleod and continued by the author. When it is crossed with 4-137a(T^L), two members of each tetrad have little tyrosinase activity. Among the producing strains, some produce T^L-tyrosinase, while others produce T^S-tyrosinase, showing

that ty-2 is independent of the T-locus, and the parental ty-2 strain, W913-17, carries the T^S allele (Table 4).

To test the genetic relation between ty-1 and ty-2, the following cross was made:

Het.B-11A(T^L,hist,ty-1)(T^S,ad,ty-1^+) X 91-151a(T^S,ty-2)

The simplest cross, ty-1 X ty-2, is not feasible, because of the female sterility of the two mutants. Although the analysis is preliminary at present, six complete asci have been tested (Table 5). The six asci all contain two histidineless and two "velvet" or ty-1 strains, showing that fertilization has occurred between (T^L,hist,ty-1) nuclei of the heterocaryon and ty-2. Among the two ty-1^+ strains of each ascus, the production of tyrosinase is segregating. This indicates that the ty-2 is not allelic with ty-1.

The forms of tyrosinase of the producing strains happened to be all T^S, which was unexpected. Further analyses are necessary before any conclusions are made on this point.

C. Heterocaryosis

Heterocaryons of the following genotypes have been formed by inoculating minimal slants with two strains with different biochemical markers, histidineless and adenineless.

Het.A (T^S,hist,+,ty-1)(T^S,+,ad,+)

Het.B (T^L,hist,+,ty-1)(T^S,+,ad,+)

Het.D (T^L,hist,+,ty-1)(T^{Sing2},+,ad,+)

Table 4. Genetic analysis of ty-2.[1)]

91: 65-811A(T^{L},ty-2^{+}) X W913-17a($T^{(S)}$,ty-2)

ascus-1		ascus-2	
91-111 ($T^{?}$,ty-2)	0[2)]	91-131 ($T^{?}$,ty-2)	2
91-112 ($T^{?}$,ty-2)	2	91-132 (T^{L},ty-2^{+})	40
91-113 (T^{L},ty-2^{+})	58	91-133 ($T^{?}$,ty-2)	0
91-114 (T^{S},ty-2^{+})	57	91-134 (T^{S},ty-2^{+})	29
ascus-3		**ascus-4**	
91-141 ($T^{?}$,ty-2)	4	91-151 ($T^{(S)}$,ty-2)	0
91-142 ($T^{?}$,ty-2^{+})	26	91-152 ($T^{(S)}$,ty-2)	5
91-143 ($T^{?}$,ty-2^{+})	164	91-153 (T^{L},ty-2^{+})	83
91-144 ($T^{?}$,ty-2)	0	91-154 (T^{L},ty-2^{+})	129

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1) The forms of tyrosinase which were deduced from the pedigree are shown in parenthesis, e.g. $T^{(S)}$ and $T^{(L)}$. Cases where no deduction is possible or not tested are designated as $T^{?}$.

2) Relative production of tyrosinase activities.

Table 5. Genetic analysis of NS68:

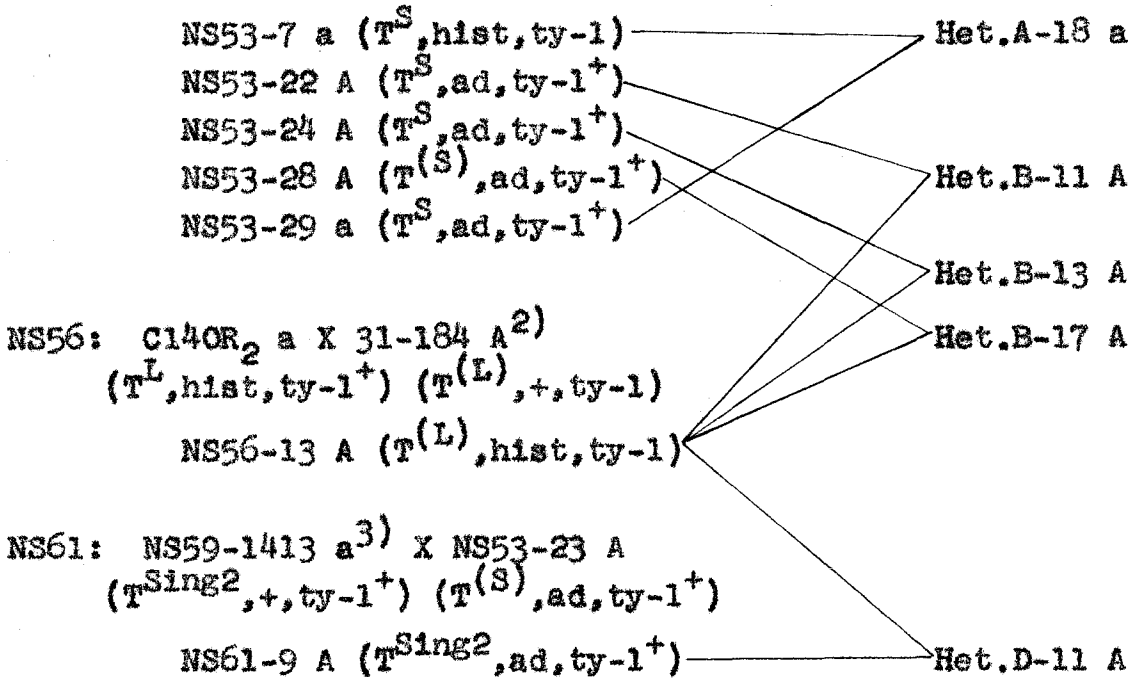
Het.B-11A(T^{L},hist,ty-1)(T^{S},ad,ty-1^{+}) X 91-151a($T^{(S)}$,ty-2).

Asci	Strains	Velvet (ty-1)	hist	Relative activity	Form of tyrosinase
ascus-1	68-511	vt	+	-	-
	68-512	vt	hist	-	-
	68-513	+	+	1	-
	68-514	+	hist	0	-
ascus-2	68-611	+	+	0	-
	68-612	+	+	51	T^{S}
	68-613	vt	hist	-	-
	68-614	vt	hist	-	-
ascus-3	68-711	vt	+	-	-
	68-712	+	hist	2	-
	68-713	+	hist	0	-
	68-714	vt	+	-	-
ascus-4	68-721	vt	+	-	-
	68-722	vt	hist	-	-
	68-723	+	+	29	T^{S}
	68-724	+	hist	130	T^{S}
ascus-5	68-731	vt	hist	-	-
	68-732	+	+	91	T^{S}
	68-733	vt	+	-	-
	68-734	+	hist	0	-
ascus-6	68-741	+	+	0	-
	68-742	+	+	43	T^{S}
	68-743	vt	hist	-	-
	68-744	vt	hist	-	-

Their pedigree is given in Fig. 8. Only the heterocaryon can grow on minimal medium. The homocaryotic components of the heterocaryons were prepared by crossing the standard strains (T^L, T^S, and T^{Sing2}) with the necessary mutants (histidineless, adenineless and ty-1) (Fig. 8). The form of tyrosinase produced by ty-1^+ strains was tested by heat-treatment and electrophoresis, while that of ty-1 strains was inferred from the pedigree.

In order to isolate stock cultures of the heterocaryons, conidia from each successful combination were inoculated on minimal agar plates and incubated overnight at 25°C. Hyphal tips were isolated and inoculated on minimal agar slants, giving stock cultures of the heterocaryons. This procedure excludes the possibility of syntrophy of the two homocaryotic strains (23). The existence of both types of nuclei and their proportion in these heterocaryons have been investigated by making reciprocal crosses between heterocaryons and a wild-type strain (4-137). A sample of random ascospores from each cross was suspended in one ml of sterile water in a small tube, heated at 60°C for 30 minutes, and then 0.25 ml aliquots of each suspension were plated on minimal, histidine-supplemented, and adenine-supplemented plates. The plates were incubated at 25°C for 12-14 hours. Three types of spores--growing, germinated but not growing, and not germinated--were counted under the microscope up to about 500 spores per plate. The results are summarized in

Figure 8. Pedigree of the available heterocaryons[1)]



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1) Forms of tyrosinase which cannot be directly tested or have not been tested are deduced from the pedigree and designated in parentheses; e.g. $T^{(S)}$, $T^{(L)}$.

2) For a further pedigree of 31-184, see Fig. 7 .

3) For a further pedigree of NS59-1413, see Fig. 5 .

Table 6. The germination was in most cases more than 95% and the proportionsof the growers and the non-growers on minimal plates were nearly 1 to 1 in all cases (Table 7), which indicates no selection against the spores of biochemical mutants in the present tests. The proportion of histidineless nuclei in a heterocaryon can be estimated from the fraction of non-growers among the germinated spores on the adenine-supplemented medium, and that of adenineless nuclei from the proportion of non-growers on the histidine-supplemented medium. In each cross these proportions add up to ca 50%. The results indicate that 1) the heterocaryons tested do contain both types of nuclei, 2) the histidineless nuclei are in all cases in excess (Table 8).

The nuclear ratios of Het.B-11, Het.B-13, and Het.B-17 have also been estimated by plating conidia on four kinds of plates; minimal (m), histidine-supplemented (h), adenine-supplemented (ad), and histidine plus adenine-supplemented (h+ad). The basic minimal medium is the same as Fries except that 20 gm of sucrose is replaced by 8 gm of L-sorbose and 1 gm of sucrose per liter. A sample of conidia from a freshly prepared slant of each heterocaryon (incubated at 25°C for six days) is added to 80 ml of sterile water, shaken vigorously, and filtered through glass wool to remove hyphal fragments. The conidial concentration was estimated by counting conidia with a hemocytometer and also by measuring the optical density in the Klett-Summerson

Table 6. Number of growing and non-growing ascospores from reciprocal crosses between heterocaryons and wild-type strain (4-137) on minimal, histidine-supplemented and adenine-supplemented media.

Crosses	Ascospores	Plates		
		minimal	+hist	+ad
HB-11 X 4-137	growers	245	433	412
	non-growers	230	72	222
	non-germinated	21	12	12
	Total	496	517	656
	% of germination	95.8%	97.7%	98.2%
4-137 X HB-11	growers	254	419	266
	non-growers	222	42	127
	non-germinated	14	13	15
	Total	490	474	408
	% of germination	97.1%	97.3%	96.3%
HB-13 X 4-137	growers	280	473	313
	non-growers	257	102	189
	non-germinated	18	21	13
	Total	555	596	515
	% of germination	96.8%	96.5%	97.5%
4-137 X HB-13	growers	315	469	452
	non-growers	301	62	323
	non-germinated	19	24	16
	Total	635	555	791
	% of germination	97.0%	95.7%	98.0%
HB-17 X 4-137	growers	282	436	314
	non-growers	266	75	213
	non-germinated	24	43	20
	Total	572	554	547
	% of germination	95.8%	92.2%	96.3%
4-137 X HB-17	growers	248	424	288
	non-growers	240	102	177
	non-germinated	39	31	18
	Total	527	557	483
	% of germination	92.6%	94.4%	96.3%
4-137 X HD-11	growers	264	472	261
	non-growers	257	13	257
	non-germinated	6	6	3
	Total	527	491	521
	% of germination	98.9%	98.8%	99.4%

Table 7. Percentage of wild, histidineless, and adenineless ascospores from crosses between heterocaryons and a wild-type strain (4-137).

Crosses	Distribution of germinated ascospores(%)			
	Wild	Histidineless	Adenineless	Histidineless + adenineless
HB-11 X 4-137	51.6%	35.0%	14.3%	49.3%
4-137 X HB-11	53.3	32.4	9.1	41.5
HB-13 X 4-137	52.1	37.6	17.7	55.3
4-137 X HB-13	51.2	41.7	11.7	53.4
HB-17 X 4-137	51.4	40.4	14.7	55.1
4-137 X HB-17	50.8	38.1	19.4	57.5
4-137 X HD-11	50.7	49.7	2.7	52.4

Table 8. Estimated nuclear proportions in heterocaryons from reciprocal crosses between heterocaryons and a wild-type strain (4-137)

Heterocaryons	Histidineless		Adenineless	
	Het.♀	Het.♂	Het.♀	Het.♂
Het.B-11	71%	78%	29%	22%
Het.B-13	68	78	32	22
Het.B-17	73	66	27	34
Het.D-11	-	95	-	5

colorimeter with the blue filter (No. 42). The relationship of the two methods is shown in Table 9. The colorimetric estimation is convenient and gives satisfactory results, so that in later experiments only this method was used. The suspension was diluted and plated to give about 100 conidia per plate, and the plates were incubated at 25°C for five to six days, until compact colonies were clearly visible. The results are summarized in Table 11. Theoretically, the numbers of heterocaryotic, histidineless homocaryotic, and adenineless homocaryotic colonies calculated from the colonies on m, h, and ad plates should add up to the number on h+ad plates. This is the case for Het.B-11, but there are some discrepancies for Het.B-13 and Het.B-17, which may suggest the presence of selection against one or both homocaryotic forms of conidia on singly supplemented media. Nevertheless, it is also clear that histidineless nuclei are the major nuclear type. The actual ratios of the two types of nuclei cannot be estimated unless the average number of nuclei per conidium ($\bar{n}$) is obtained by counting them cytologically (24). However, the values of $\bar{n}$ have been known in macroconidia of Neurospora to be between 2 and 3 in most cases (25,26). If Atwood and Mukai's* formula is

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* $$p \approx \frac{r(1 - r) + a(\bar{n} - 2r)}{\bar{n}(1 - r)}$$

where p is the proportion of type A nuclei (hist), r is the proportion of heterocaryotic conidia, a that of type A homocaryotic conidia (hist), and $\bar{n}$ is the average number of nuclei per conidium (24).

Table 9. Relationship between conidial concentration and turbidity.

Heterocaryons	Hemocytometer counting (per ml)	Colorimeter[1] reading	cells / colorimeter unit
HB-11 (1)	4.6×10^5	7	66,000
(2)	5.1×10^5	7	73,000
HB-13	3.6×10^5	6	60,000
		Ave.	66,000

1) Klett-Summerson colorimeter was used with the blue filter (No. 42).

Table 10. Plating of conidia of heterocaryons on minimal, histidine, adenine, and histidine plus adenine media.

Heterocaryons	Average number of colonies per plate				Number of plates used for each average
	min	+hist	+ad	+hist+ad	
Het.B-11 (1)	12.5	25.0	18.0	30.3	3
(2)	19.7	51.5	26.3	59.8	
Het.B-13	23.8	62.3	34.0	91.3	4
Het.B-17	26.5	63.5	38.8	100.8	4

Table 11. Analysis of Table 10.

Heterocaryons	Number and (%) of colonies per plates				Number of colonies on hist+ad plate
	het.[1] (r)	hist-homo.[2] (a)	ad-homo.[3] (b)	Total	
Het.B-11 (1)	12.5 (41)	12.5 (41)	5.5 (18)	30.5 (100)	30.3
(2)	19.7 (34)	31.8 (55)	6.6 (11)	58.1 (100)	59.8
Het.B-13	23.8 (33)	38.5 (53)	10.2 (14)	72.5 (100)	91.3
Het.B-17	26.5 (35)	37.0 (49)	12.3 (16)	75.8 (100)	100.8

1) Number of colonies on the minimal plate.

2) Number of colonies on the histidine plate minus that on the minimal plate.

3) Number of colonies on the adenine plate minus that on the minimal plate.

applied to the present cases for $\bar{n} = 2$ and 3, the frequencies of histidineless nuclei are between 62 and 76 per cent (Table 12), which agrees fairly well with those values estimated by plating ascospores (Table 14).

The growth rate of some heterocaryons have been compared on 400-mm horizontal growth tubes (27). The growth rates of heterocaryons, Het.A and Het.B, on minimal medium were the same as that of a standard wild type, 4-121. This suggests that the observed range of nuclear ratios is within the optimal complementation of histidineless and adenineless nuclei (23). Het.D, however, showed a lag of growth and a slower rate of growth both on minimal and histidine- or adenine-supplemented media. The explanation may be the incomplete compatibility of the two component strains of Het.D rather than the insufficiency of adenineless nuclei (actually 5%) for metabolic complementation.

The tyrosinase production of the heterocaryons and their component strains has been tested, and the results are summarized in Table 13. None of the component ty-1 strains produce any detectable amount of tyrosinase, and the ty-1^+ strains produce 20 to 60 per cent as much as standard strains 4-121 and 4-137. The low production of the mutant strains may be due to the culture conditions of these mutants, e.g. the amount of supplementation of histidine or adenine and the culture period (four days), or to their genetic background. In fact, the adenineless

Table 12. Estimation of the proportion of histidineless nuclei in heterocaryons as a function of $\bar{n}$ [1] from the data in Table 9.

$\bar{n}$	Het.B-11 (1)	Het.B-11 (2)	Het.B-13	Het.B-17
1	53%	61%	64%	58%
2	62	72	69	67
3	64	76	73	70
4	66	78	74	71

1) Calculations were made using observed values of r and a (Table 9) for the equation,

$$p \approx \frac{r(1-r) + a(\bar{n}-2r)}{\bar{n}(1-r)}$$

Table 13. Relative production of tyrosinase in heterocaryons and their related strains.

Strains	Genotypes	Relative tyrosinase production
Het.A-18	(NS53-7a)(NS53-29a)	45
Het.B-11	(NS53-22A)(NS56-13A)	19
Het.B-13	(NS53-24A)(NS56-13A)	23
Het.B-17	(NS53-28A)(NS56-13A)	24
Het.D-11	(NS56-13A)(NS61-9A)	37
NS53-7a	(T^S,hist,ty-1)	0
NS53-22A	(T^S,ad,ty-1^+)	48
NS53-24A	(T^S,ad,ty-1^+)	30
NS53-28A	(T^S,ad,ty-1^+)	-
NS53-29a	(T^S,ad,ty-1^+)	19
NS56-13A	(T^L,hist,ty-1)	0
NS61-9A	(T^{Sing2},ad,ty-1^+)	55
4-121A	(T^S)	100
4-137a	(T^L)	100
3520$\frac{3}{3}R_2$	(T^S,ad,ty-1^+)	23
C140R_2	(T^L,hist,ty-1^+)	63

strains do not give any tyrosinase activity on supplemented medium with 1 mg of adenine HCl when cultured for four days, but they produce 20 to 60% as much as the standard strains when cultured six days.

The heterocaryons tested produce tyrosinase in amounts of 20 to 50 per cent of the standard strains. These results show that the ty-1 allele is recessive to its normal form, ty-1^+, when they are brought together in the same cell. There is no clear correlation between the yield of enzyme and the proportion of ty-1^+ component. Now the question is whether the enzyme produced by a heterocaryon is 1) the form which the ty-1^+ component of the heterocaryon produces in the homocaryotic state, 2) a mixture of the two forms, determined by the alleles of the T-locus present in the heterocaryon, or 3) an entirely new form or forms of the enzyme. The answer to these questions turned out to be the second one; namely two forms of tyrosinase were produced in simple mixture, and these were the two forms determined by the T-alleles present in the heterocaryons. The identification of the tyrosinase forms has been made by thermal inactivation and electrophoresis of extracts of the heterocaryons.

The tyrosinase of Het.A-18 which has the genotype (T^S,hist,ty-1)(T^S,ad,ty-1^+) showed a first order curve in heat-inactivation with a half-life of 40 min in a crude, and 70 min in a partially purified preparation (Figs. 9 and 10), and a single band of T^S-mobility in the zone electrophoresis (Fig. 11), indicating that in spite of the total

Figure 9. Heat-inactivation curves of partially purified T^S-, T^L-, and Sing-2 forms of tyrosinase at 59°C. (0.1 M Na-phosphate buffer, pH 6.0).

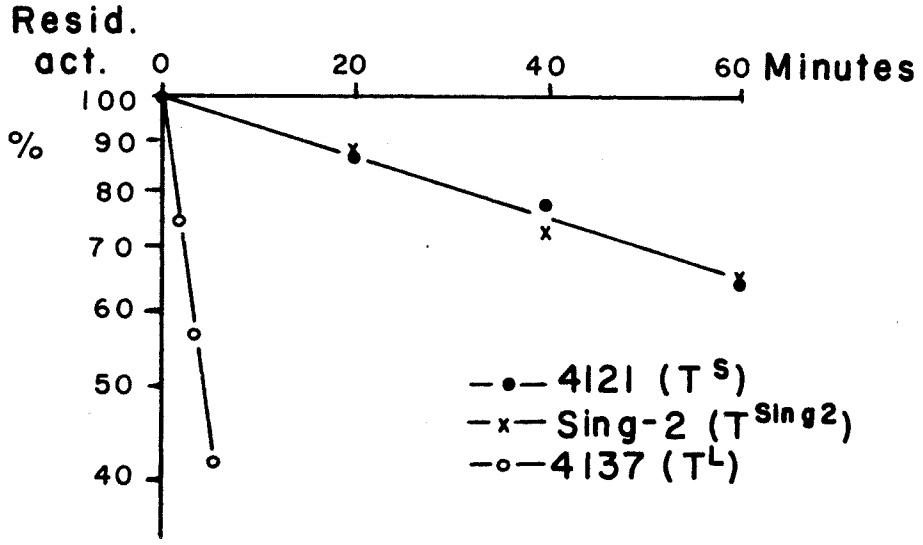
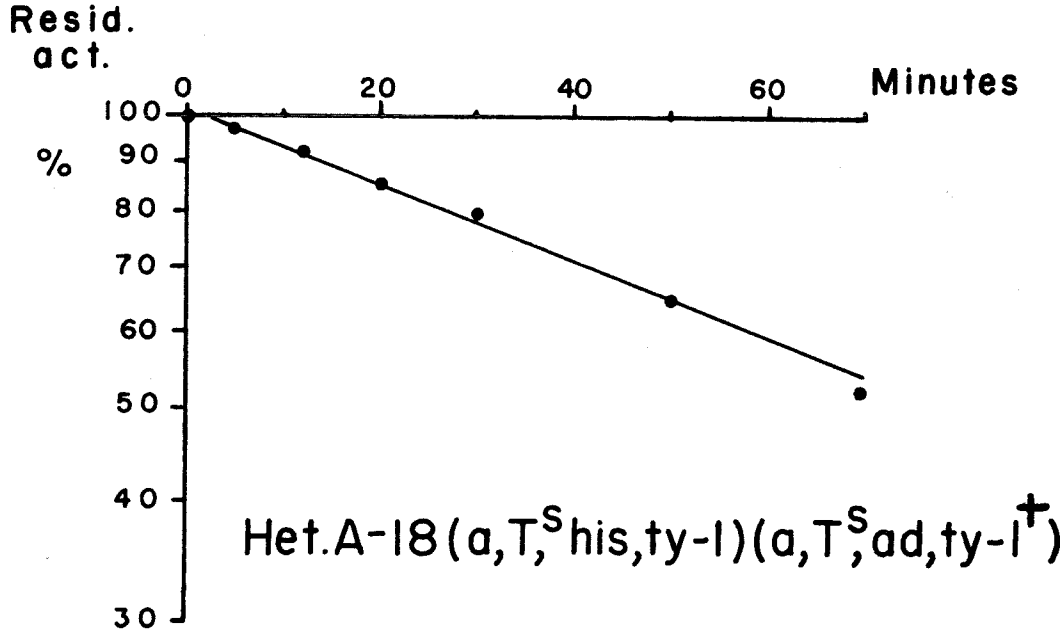


Figure 10. Heat-inactivation curve of partially purified tyrosinase from Het.A-18 at 59°C. (0.1 M Na-phosphate buffer pH 6.0).



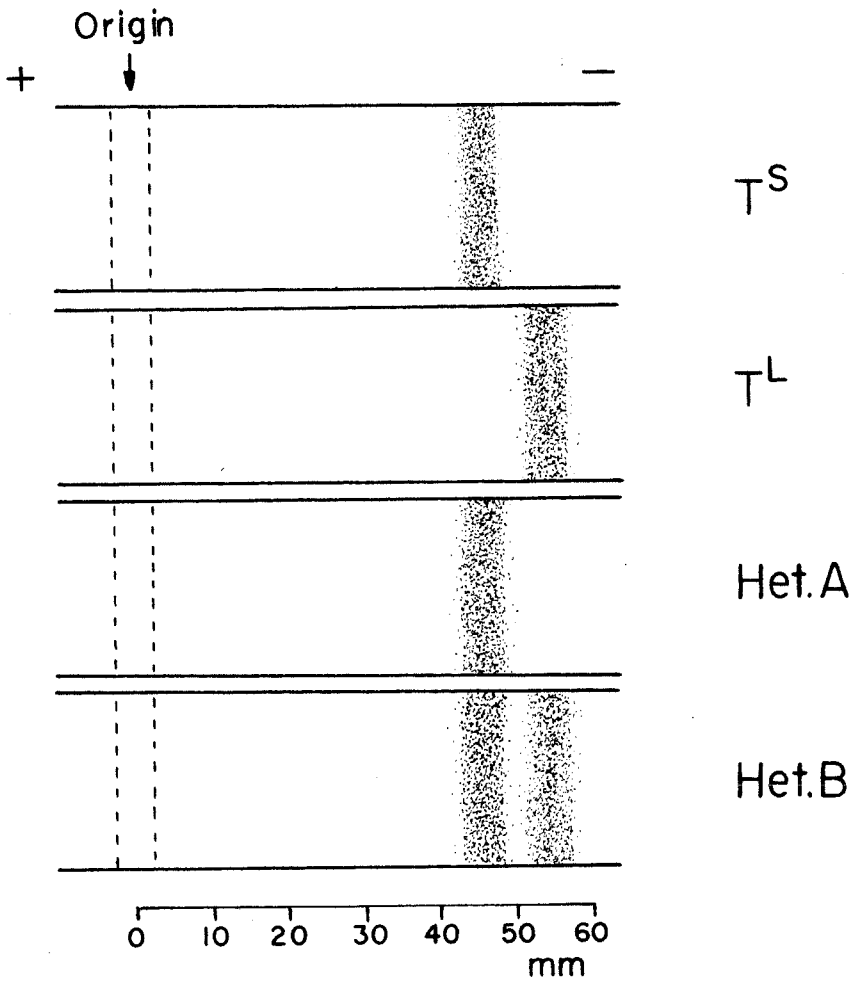


Figure 11. Electrophoretic pattern of tyrosinase of T^S, T^L, Het.A and Het.B strains. (0.05 M Na-phosphate buffer, 1.25 A per strip for 35 hours).

blocking of tyrosinase in homocaryons, the ty-1 allele does not function in the heterocaryon either in production or in affecting the structural properties of tyrosinase molecules.

The crude and partially purified enzyme preparations of the three strains of Het.B and Het.D gave a marked bending curve in thermostability, which suggests the presence of two forms of tyrosinase, heat-labile and heat-stable (Figs. 12, 13 and 14). This has been confirmed in the three strains of B type heterocaryons by electrophoresis (Fig. 11). They gave two bands in paper electrophoresis with T^S and T^L migrations, respectively. The thermostability curves made it possible to estimate the amount of each form of tyrosinase on the assumption that the labile component has a half-life of five minutes and the stable one of 70 min. The validity of these assumptions is shown by the excellence of the agreement between calculated and observed thermal inactivation curves (Figs. 13, 14). The amounts of T^S- and T^{Sing2}-forms at zero time were estimated by extrapolating the straight part (representing respectively the inactivation of the stable component, T^S or T^{Sing2}) of the curve to time zero, and that of the T^L-form by difference.

When the dilution method was used, the first sample was taken one or two minutes after the addition of enzyme into the buffer (i.e. after true zero time) and a correction for this was made by equation 6 both for T^S or T^{Sing2} ($t_{½}$ = 70')

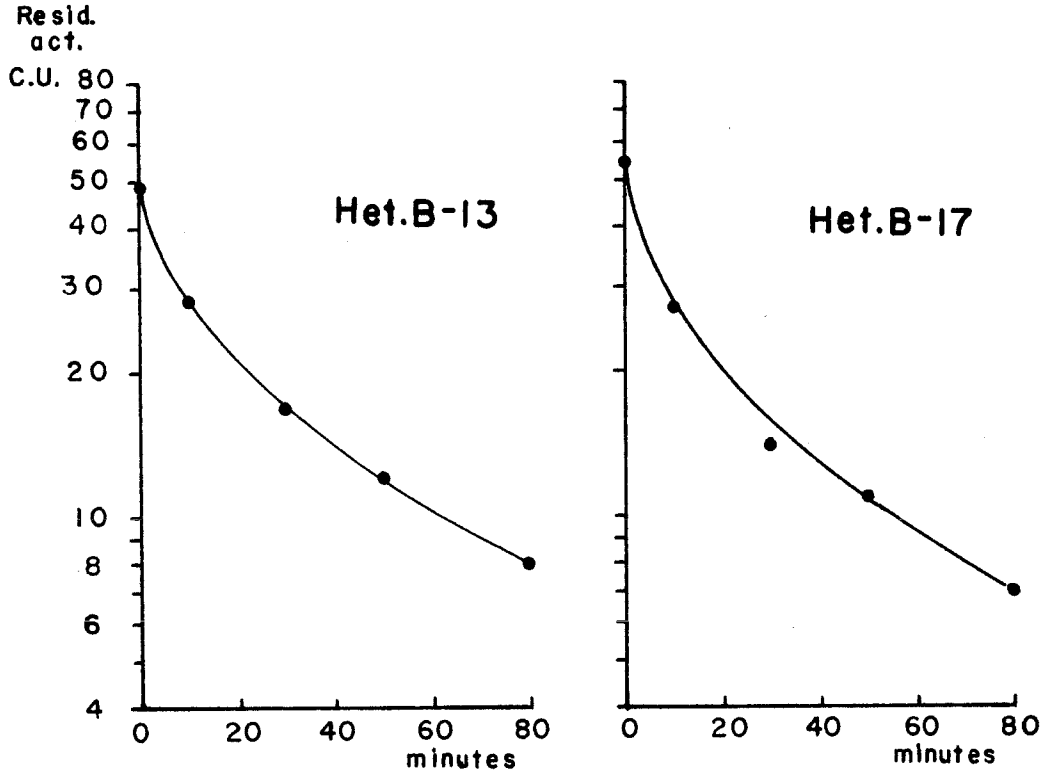
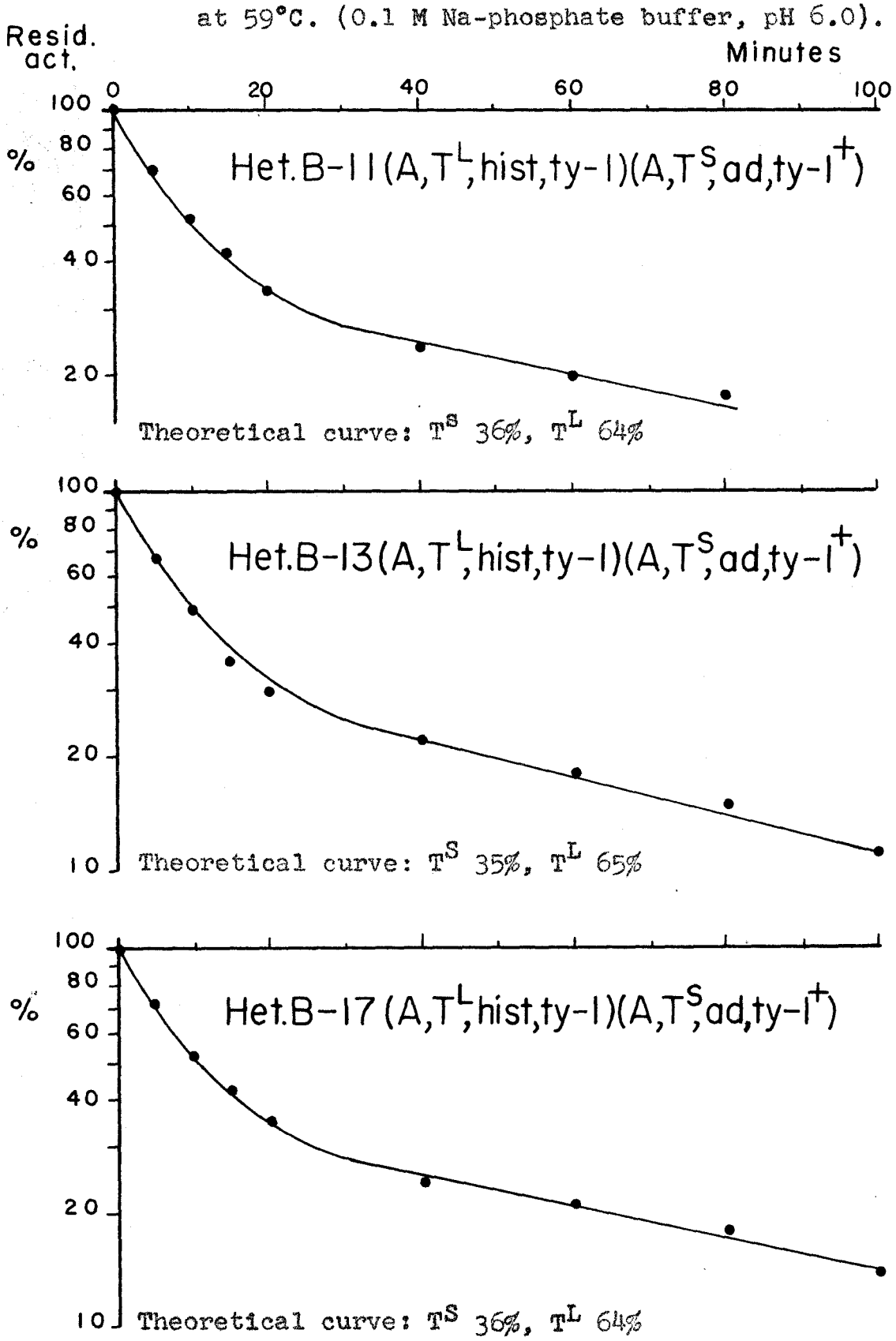


Figure 12. Heat-inactivation curve of a crude tyrosinase preparation from Het.B-13 and Het.B-17 at 59°C. (0.1 M Na-phosphate buffer, pH 6.0).

Figure 13. Heat-inactivation curves of partially purified tyrosinase from three heterocaryons of Het.B type at 59°C. (0.1 M Na-phosphate buffer, pH 6.0).



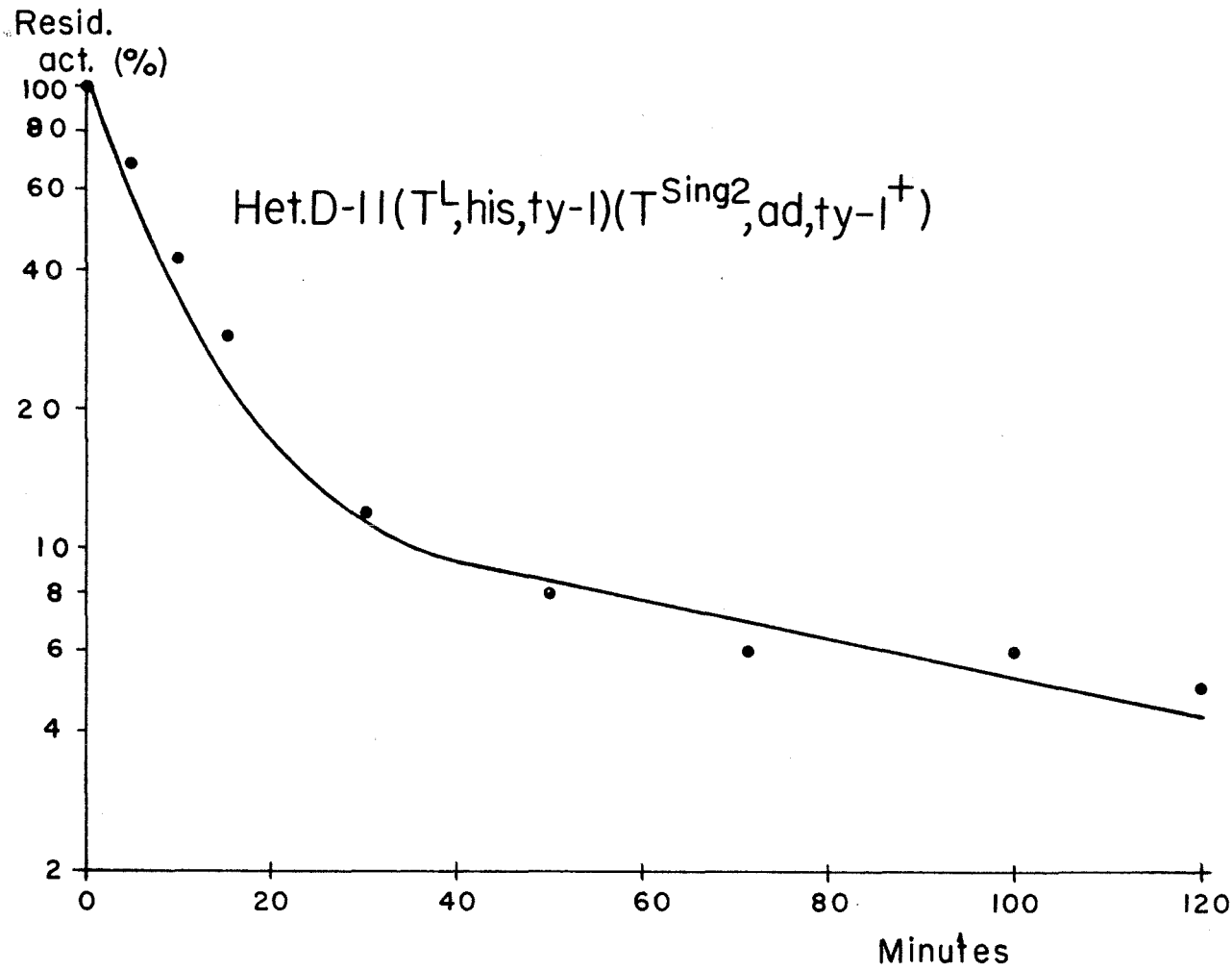


Figure 14. Heat-inactivation curve of partially purified tyrosinase of Het.D-11 at 59°C (0.1 M Na-phosphate buffer, pH 6.0).
The curve is the theoretical for a mixture containing 14 per cent of tyrosinase of half-life 70 min. and 86 per cent of half-life 5 min.

and T^L form ($t_{1/2}$ = 5').

$$\ln \frac{A_\tau}{A_0} = - k\tau = - \frac{0.693}{t}\tau \qquad (6)$$

$$A_0 = A_\tau \times e^{\frac{0.693}{t}\tau}$$

τ: time between the addition of enzyme into buffer and the drawing of the first sample.
A: activity.

The correction can also be made graphically.

The estimates of the component forms of the enzyme are given in Table 14. The comparison of the ratios of the two forms with the ratios of histidineless and adenineless nuclei is most interesting. There is an agreement in that the major nuclear type is (T^L,hist,ty-1) and the major form of tyrosinase is the T^L-form. It would be very surprising if this correlation is a mere coincidence, because the four different heterocaryons tested show a similar positive correlation. Moreover, the ty-1 allele which blocks tyrosinase production by itself is in the nuclei of the major type in all cases, and yet the prevailing form of the enzyme produced is the T^L-form; namely the type of the same nucleus.

Table 14. Proportion of T^L-tyrosinase produced by heterocaryons (estimated from thermal inactivation curves), and proportion of histidineless nuclei.

Heterocaryons	Hist-nuclei in mycelia %			T^L-tyrosinase %	
	Estimated from ascospores of reciprocal crosses		Estimated[1] from conidia	Estimated from heat-stability curves	Electrophoresis
	Het.♀	Het.♂			
Het.A-18 $(T^S,hist,ty\text{-}1)(T^S,ad,ty\text{-}1^+)$	-	-	-	0	T^S
Het.B-11 $(T^L,hist,ty\text{-}1)(T^S,ad,ty\text{-}1^+)$	71	78	72 - 76	67	T^L and T^S
Het.B-13 $(T^L,hist,ty\text{-}1)(T^S,ad,ty\text{-}1^+)$	68	78	69 - 73	69	T^L and T^S
Het.B-17 $(T^L,hist,ty\text{-}1)(T^S,ad,ty\text{-}1^+)$	73	66	67 - 70	67	T^L and T^S
Het.D-11 $(T^L,hist,ty\text{-}1)(T^{Sing2},ad,ty\text{-}1^+)$	-	95	-	88	T^{Sing2} not detectable

1) The ranges were calculated by Atwood and Mukai's formula (24) for $\bar{n}$ (average number of nuclei per conidium) of 2 and 3.

IV. DISCUSSION

A. Genetic considerations

The following facts have been obtained in the present investigation.

(1) A new form of tyrosinase, Singapore-2, has been characterized. This form differs from the known three forms, T^S, T^L, and T^{PR15}, electrophoretically and in thermostability.

(2) The characteristics of the Sing-2 tyrosinase are apparently determined by the T-locus, which is also responsible for the other tyrosinase forms mentioned. These tyrosinases thus form an allelic series.

(3) Strains carrying the gene ty-1 produce no tyrosinase in most cases, while a small amount is produced in a few ty-1 derivative strains. The locus of ty-1 is independent of the T-locus, and ty-1 has no specificity for T^S or T^L, i.e., it is epistatic to both. The absence of tyrosinase activity in strains carrying ty-1 is not due to the presence of an inhibitor (8). Another gene, ty-2, which also causes tyrosinase deficiency seems to occupy an independent locus from the T and ty-1 loci.

(4) Heterocaryons of the following genotypes have been produced:

Het.A $(T^S, hist, +, ty\text{-}1)(T^S, +, ad, ty\text{-}1^+)$

Het.B $(T^L, hist, +, ty\text{-}1)(T^S, +, ad, ty\text{-}1^+)$

Het.D $(T^L, hist, +, ty\text{-}1)(T^{Sing2}, +, ad, ty\text{-}1^+)$

Since the discovery of an abnormal human hemoglobin by Pauling et al. in 1949 (28), a number of examples of gene controlled modifications of protein molecules have been reported. Table 15 summarizes the available data. The detailed chemical differences between the variant forms of protein have been investigated in human hemoglobins, A, S, and C (29,30,31,32) and it has been found that the differences are changes in but a single aminoacid residue in the half molecule, namely a glutamic acid (A) to valine (S), and to lysine (C). Thus in this case it was suggested that a single mutation resulted in a single aminoacid change.

Although genetic analyses are not feasible, interspecific differences in the aminoacid sequence are found in insulin (49), cytochrome C (50), vasopressin (51), and ACTH (52), among different species of vertebrates.

The four forms of Neurospora tyrosinase are likely to show similar modifications in aminoacid sequence. In this sense, the T-locus could be regarded as the primary gene which determines the basic structure of the tyrosinase molecule (10,14,53). Actual amounts of tyrosinase produced depend, however, upon several other factors, both hereditary and environmental. It has been known that the content of sulfur in the medium, temperature and air supply of the culture influence the quantitative production of the enzyme (14). As for the genetic blocking of the tyrosinase production, two non-producer genes, ty-1 and ty-2, have been

All produce 20% to 50% as much tyrosinase as the standard strains.

(5) Het.A produces T^S-form of tyrosinase only. Three heterocaryons of type B produce a mixture of both forms of the enzyme. The T^L-form is in excess of T^S-form in the three B-type heterocaryons tested (T^L-form: 67-69%). Het.D produces both T^L- and Sing2- forms in mixture. Again T^L-form is in excess (88%).

(6) The ratios of the histidine and the adenine nuclei in the heterocaryons have been estimated a) by plating ascospores from the cross between heterocaryons and wild type onto minimal, histidine-, and adenine-supplemented plates, and b) by plating conidia of heterocaryons onto minimal, histidine-, adenine-, and histidine plus adenine-supplemented plates. The results show that in all heterocaryons tested the histidine nuclei are the majority type (66-95%). Thus the ratios of the two forms of tyrosinase in Het.B and Het.D correspond to that of the two kinds of nuclei.

These results permit certain conclusions concerning the relationship between gene and enzyme. Simple Mendelian segregations obtained in crosses involving the structurally different forms of tyrosinase can be best interpreted on the basis of a single locus (T-locus) which determines the structure of the molecule. The fact that all four types--of widely different geographical origins--are inherited as alleles suggests that the T-locus is the only locus that controls the enzyme structure.

Table 15. Some single gene modifications of protein structure

Protein	Organism	Form	Properties	References
Hemoglobin	human	A, S, C	Elect.mobil	33,34
	cattle	A,B	Elect.mobil.	35,36
	goat	2 forms	Elect.mobil.	37
	sheep	Hb-I,Hb-II	Elect.mobil	38-41
	mouse	2 forms	Elect.mobil.	42,43
β-Lactoglobulin	cattle	α,β	Elect.mobil.	44
Tyrosinase	Neurospora	T^S,T^L,T^{Sing2},T^{PR15}	Elect.mobil. Thermostability	7,12 Present thesis
Pantothenate synthesizing enzyme	E. coli	2 forms	Thermostability	45
Glutamic acid dehydrogenase	Neurospora	2 forms	Temperature response	46
Adenylosuccinase	Neurospora	2 forms	Thermostability	47
Pyrroline-5-carboxylate reductase	Neurospora	2 forms	Thermostability	48

found (54). The strains which carry these alleles can grow on minimal medium as rapidly as tyrosinase producing strains, indicating the non-essentiality of tyrosinase for vegetative growth of Neurospora. The defect of the mutants is, however, manifested in sexual processes. Both ty-1 and ty-2 are female-sterile, that is, they do not form protoperithecia, while the conidia are perfectly functional in fertilizing protoperithecia of wild type. This genetic inhibition of enzyme production is analogous to those cases of tyrosinase in Glomerella (55), of β-galactosidase in Neurospora (56), of laccase in Neurospora (57), and of xanthine dehydrogenase in Drosophila (58); namely, more than one gene is responsible for the production of one enzyme. None of these cases could, however, provide any critical evidence on the primary function of genes in the enzyme formation, simply because of the absence of functional distinctions between the genes involved. One possibility is that the mode of participation of these genes in enzyme formation may be indirect (55,59), modifying internal physiology of the cell. Another possibility is that each gene is responsible for the production of a part of the enzyme molecule. Thus each component polypeptide chain in an enzyme molecule may be controlled by a locus, or the synthesis of the prosthetic group of the enzyme may be controlled by a series of biochemical steps, corresponding to a series of loci. These possibilities do not explain the apparent specificity, however,

One striking feature of these multi gene-one enzyme cases is that they have been clearly demonstrated only in enzymes with no indispensable function for growth in minimal medium. Among indispensable enzymes, no case has been reported in which more than one chromosomal region blocks the formation of a single enzyme, but independent mutants deficient in an enzyme have been shown to have mutations in a single chromosomal region (60-64), with a possible exception of hemoglobin in man (thallassemia) (5). This, of course, does not exclude the presence of modifier genes for enzyme formation. The existence of modifying genes has been found in a number of instances; for example, several suppressor genes bring about the restoration of the enzyme in allelic mutants deficient in tryptophan synthetase (60,64), and a genetic variation affects the "residual enzyme level" in leakage of proline-requiring allelic mutants (59).

From the present results of heterocaryon experiments, it is clear that ty-1 is recessive in nature; and, furthermore, extracts of ty-1 mycelium do not show any inhibitory effect on tyrosinase activity. This suggests that the abnormality of ty-1 strains is the lack of enzyme rather than the accumulation of inhibitors. It is interesting to note that ty-1 is, therefore, epistatic to the T-locus and yet recessive to its allelic form ($ty\text{-}1^+$). Moreover, ty-1 acts through cytoplasm, because heterocaryons of type (T^L,ty-1)(T^S,$ty\text{-}1^+$) produce not only T^S-form but also T^L-form.

A heterocaryon of $(T^S)(T^L)$ has been reported (9) and it was found that it produces both forms of the enzyme in simple mixture, without forming hybrid molecules. This is analogous to the hemoglobin case in which heterozygotes of Hb^A/Hb^S, Hb^A/Hb^C, Hb^S/Hb^C types produce the mixtures of the respective two forms, and differs from the complementary interaction of adenylosuccinase (47) and glutamic acid dehydrogenase (65) in allelic Neurospora mutants.

The correlation between the nuclear ratio and production of the corresponding enzymes can hardly be fortuitous, for four tested heterocaryons give similar positive correlation. The nuclear ratio has been tested by plating both ascospores and conidia with similar results, which indicates the real nuclear frequencies in the mycelium are near the ratios of the two nuclear types actually found. Whether this unequal frequency comes from a physiological balance of the cell concerning the histidine and adenine production (66), or from the unequal nuclear frequency in the originally fused hyphae (67) cannot be decided.

These facts can be interpreted by the following hypotheses: The primary product (T-product) of the T-locus carries specific structural information relating to the tyrosinase molecule, probably an aminoacid sequence. The $ty\text{-}1^+$ allele, the $ty\text{-}2^+$ allele, and probably other undiscovered genes are required to provide the right conditions for the functioning of the T-product in tyrosinase synthesis. Thus in hetero-

caryons, two forms of the T-product are in mixed population in the cell and ty-1^+ provides non-specific factors necessary for tyrosinase production. The nature of the T-product is of course speculative. However, the possibility that the T-product is protyrosinase (an inactive similar protein) is not very probable, for the ty-1 strain does not contain serologically cross-reactive material (8). Another possibility is that the T-product is not a related protein but some other component of the tyrosinase synthesizing system, for example, microsomes. The ty-1 type genes, with indirect functions in enzyme formation, may be numerous, and indeed in Neurospora tyrosinase a second gene, ty-2, is found to have an entirely different locus. The possibility that these secondary genes in tyrosinase production are involved in copper metabolism is an attractive one, but there is no evidence for it.

The one gene-one enzyme hypothesis is formally incorrect in most of the cases of enzyme production where multigenic modifications are found. However, the basic question is--what is the primary function of the gene? If the primary function of the gene is to convey structural information to the protein molecule (template theory), as a logical sequence, there must be one gene which acts as the template for a specific protein or polypeptide chain. In this sense the one gene-one enzyme theory is valid also in the present case. Only one locus has been found in Neurospora which affects specifically the structure of tyrosinase and two

others affect quantitative aspects of the production of the enzyme. Thus each nucleus of the heterocaryon produces a primary product of its T-locus and this product has the information for the structure of the tyrosinase molecule. The actual synthesis of tyrosinase, however, may be several steps removed from the gene.

B. Some considerations regarding thermostability and electrophoretic behavior of Neurospora tyrosinase

Thermostability

Thermostability of proteins is in general dependent on various factors: pH; ionic strength; kinds of ions; presence of substrates, other proteins, inhibitors, and other substances; enzyme concentration; etc. Neurospora tyrosinase is no exception (18, 9). The conditions for measuring thermostability have been made as uniform as possible. However, the macromolecular contaminants in the preparations are not easy to remove with any reproducible pattern even by the same procedure of partial purification, because of the strain differences in the initial crude extracts. Thus partially purified preparations of Sing-2 tyrosinase from various strains give half-lives of 35 to 110 minutes at 59°C in 0.1M Na-phosphate buffer pH 6.0, although the electrophoretic migration is the same for all. Even different preparations from the same strain give different stabilities which cannot be attributed to experimental errors of heat treatment. There

is clearly, however, less intra-strain variability than inter-strain variability. This variation did not disturb the characterization of the segregation within each ascus in crosses between T^{Sing2}, T^{L}, and T^{PR15} because of the large difference in their stabilities. This large variation at 59°C seems, however, to decrease in a higher temperature range. Arrhenius plots of the data (Figs. 2, 3, and 4) show that the three forms of Neurospora tyrosinase give a similar pattern of inactivation kinetics. A striking feature of the kinetics is that there is a discontinuity in Arrhenius plots for the three forms; namely at about 61°C for Sing-2 and T^{S}-forms and at about 56°C for the T^{L}-form. The only detailed thermal inactivation study of an enzyme in the absence of substrate which the author could find in the literature is the case of beef liver catalase (17). This case also shows a definite break in Arrhenius plots. Whether this is a general phenomenon for thermal denaturation of protein or not is not clear. Rate changes of enzyme catalyzed reactions with a discontinuity of Arrhenius plots are known in several instances (68). A number of explanations for such cases has been suggested.

Some possible explanations for the present case will be described.

(1) Two parallel reactions, e.g. at different sites on the enzyme with different temperature coefficients, may be involved in the inactivation. The rate of the reaction

with a higher coefficient increases more rapidly with temperature than the other, and therefore this reaction predominates at higher temperatures. It would, therefore, be expected that the higher activation energy would be that observed at the higher temperatures and the Arrhenius plots should be concave upwards, which is true in the present case and in the catalase case.

(2) The enzyme may exist in two forms with different activation energies of denaturation. If the enzyme exists in two forms in equilibrium with one another, both forms being active but having different activation energies of inactivation and if the effect of temperature on the change from one form to the other is large, a sharp discontinuity will be observed.

Beside these alternative explanations, another feature should be noticed in the case of T^S- and Sing2-tyrosinase forms, namely that the inactivation reaction with the lower activation energy is modified greatly by other factors in the preparation, causing a relatively large variation in the stability of different preparations.

Electrophoretic migrations

The methods described in this paper for electrophoresis of Neurospora tyrosinase were developed by Horowitz and Fling (personal communication). The mobility is quite reproducible. Crude extracts from mycelia give migrations

identical to those of the purified preparations, and this has facilitated the analysis of a large number of strains in genetic experiments.

Differences in mobility between Sing2-form and T^S- or T^L-form have been confirmed by electrophoresis of mixtures of the two forms. The electrophoresis of the mixture separates the two components, giving two bands corresponding to those of the component forms. The two component forms of tyrosinase (T^S- and T^L-forms) from Het.B have been also separated by electrophoresis.

The reason for the appearance of more than one band among a few T^{Sing2}-segregants of the cross, $T^{Sing2} \times T^L$, is not clear. The phenomenon is, however, not reproducible and the extra bands are extremely labile; for example, keeping the crude extract in the freezer, purification, dialysis against 0.1M phosphate buffer, pH 6, and brief heating lead to the disappearance of the extra bands.

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PART II

I. INTRODUCTION

In their study of Neurospora tyrosinase, Horowitz and Fling (1) described the presence of a "second phenol oxidase" in the culture of N. crassa which had several different characteristics from tyrosinase. The enzyme rapidly catalyzed the oxidation of DL-DOPA but had little effect on the oxidation of tyrosine. The enzyme was more thermostable than any forms of the tyrosinase which they had been working on; the production of the enzyme was higher when Neurospora was cultured at 35°C than at 25°C; and the enzyme was found chiefly in the culture medium.

Studies on this enzyme were continued by Stadler (2). On the basis of its substrate specificity and the spectrum of inhibitors, the "second phenol oxidase" was tentatively identified as a laccase. Two forms of Neurospora tyrosinase, thermostable (T^S) and thermolabile (T^L), were known to be determined by a single locus. The possibility that this locus also determined the thermostability of the laccase was examined, but no evidence was found for this.

Further studies on this enzyme have been carried out by the author in order to look for structural variations of the enzyme and study their genetic relationship. The general importance of the problem has been discussed in the first part of the present thesis. The enzymology of laccase has been discussed in several reviews (3,4) and a brief summary

will be presented here.

Laccase was first observed by Yoshida in 1883 (5) from the latex of the Japanese lacquer tree, and named as such by G. Bertrand (6) working on the enzyme from the latex of the Indo-Chinese lacquer tree. In 1939 Keilin and Mann (7) partially purified it and showed that the enzyme contained copper as the prosthetic group. This point was further supported by Tissieres (8) who split the copper from laccase by cyanide and restored the enzymatic activity by adding copper.

The enzyme occurs widely in plants: e.g. potatoes, sugar beets, apples, cabbages, various mushrooms, etc. An enzyme which has similar substrate specificity has been found in human serum and called ceruloplasmin (9).

Laccase from lacquer trees catalyzes the aerobic oxidation of various polyphenols and related compounds such as guaiacol, hydroquinone, catechol, pyrogallol, p-phenylene diamine and Nadi reagent, but does not oxidize monophenols such as tyrosine, p-cresol, or resorcinol. The activity of the purified laccase is inhibited by potassium cyanide, hydrogen sulfide, sodium azide and diethyldithiocarbamate. However, carbon monoxide is not effective on the enzyme.

II. EXPERIMENTS

A. Assay of laccase activity

The enzyme activity was assayed colorimetrically using DL-DOPA or guaiacol with the same procedure as for tyrosinase described in Part I of the present thesis. The final concentration of DOPA and guaiacol was 0.004M. Phosphate buffer, 0.1M, pH 6.0, was used throughout the experiments, unless otherwise notified. The relation between the amount of enzyme and the rate of reaction is linear in the range of 0 to 150 colorimeter units per 5 minutes for both substrates. Guaiacol has the following advantages over DL-DOPA as the substrate for laccase: it is about nine times as sensitive as DL-DOPA; it is quite stable at pH 6.0 and can be kept for weeks in the refrigerator without appreciable autoxidation; Neurospora tyrosinase has no effect on the oxidation of this compound.

B. Production of laccase

Twenty ml of the culture medium is dispensed in each 125 milliliter Erlenmyer flask, autoclaved, inoculated with conidia and incubated at 35°C for nine to twelve days. Amounts of sulfur and copper in Fries minimal medium were varied and optimal amounts for the production of laccase (100 mg of $MgSO_4 \cdot 7\ H_2O$ and 0.5 mg of Cu per liter) were used for later work. Oxidation of L-tyrosine by 11-days-old culture medium is none or little, which indicates the presence

of little tyrosinase on the medium.

The mode of enzyme appearance during the incubation is shown in Fig. 1. The growth of Neurospora and pH change of the medium are given in Fig. 2. Laccase appears in the medium on the sixth day of incubation and increases for several days, while the dry weight of mycelium starts to decrease on the fifth or sixth day. The hydrogen ion concentration of the medium also begins to decrease rapidly on the sixth day. The enzyme production of various strains was tested and is shown in Table 1.

C. Purification

Partial purification of Neurospora laccase was achieved through ethanol and ammonium sulfate fractionations. Twelve days old cultures were filtered to remove mycelia, and the collected medium was concentrated in one of two ways. First, one liter of the medium was put in a five-liter flask and concentrated under reduced pressure in a water bath (55°-60°C) to about 100 ml. A considerable amount of precipitation occurred, but all laccase activity remained in the dark brown supernatant. Sixty-six gm of solid ammonium sulfate (90% saturation) was added and the precipitate was centrifuged down and dissolved in ten ml of buffer. This method was used for larger amounts of medium (more than one liter). Second, 100 ml of the medium was put into a dialysis bag and dialyzed overnight at room temperature against 500 ml of saturated ammonium sulfate solution with surplus of

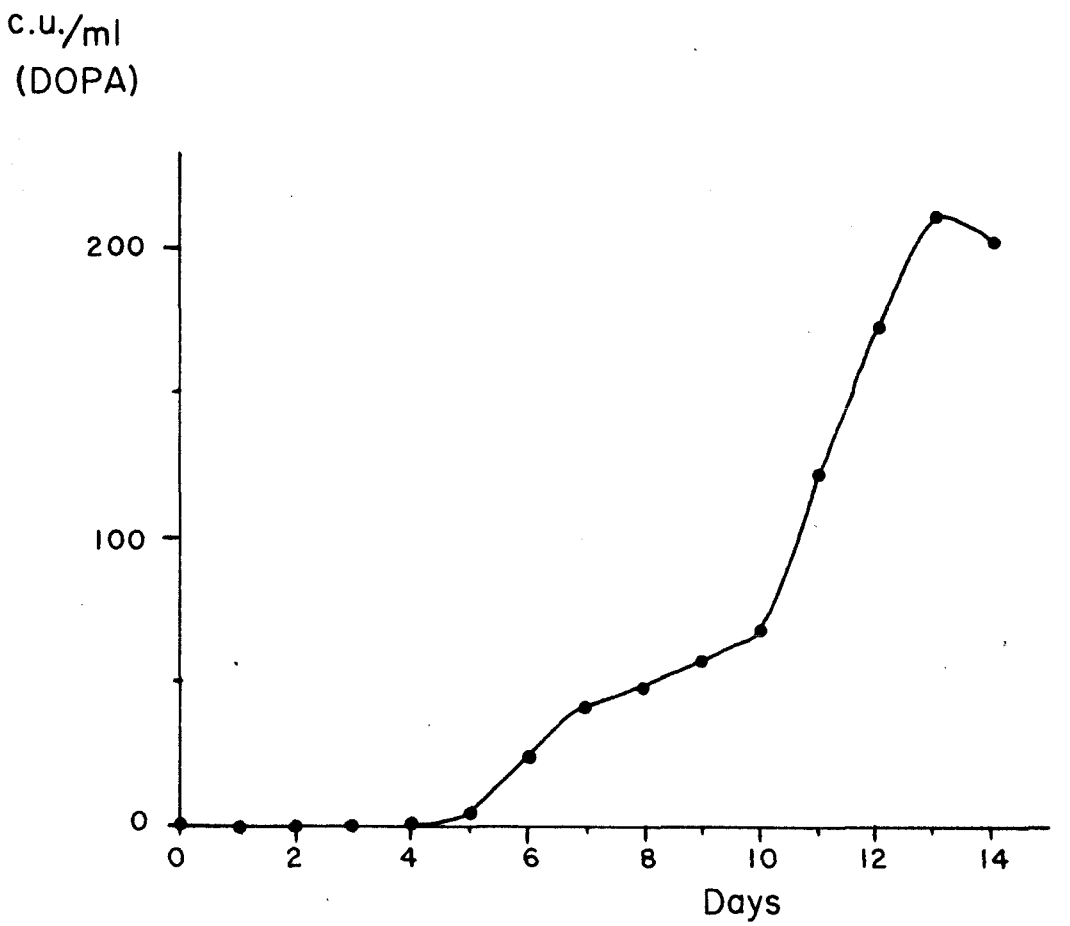

Figure 1. Laccase production of NS16-612.
(For culture conditions, see text.)

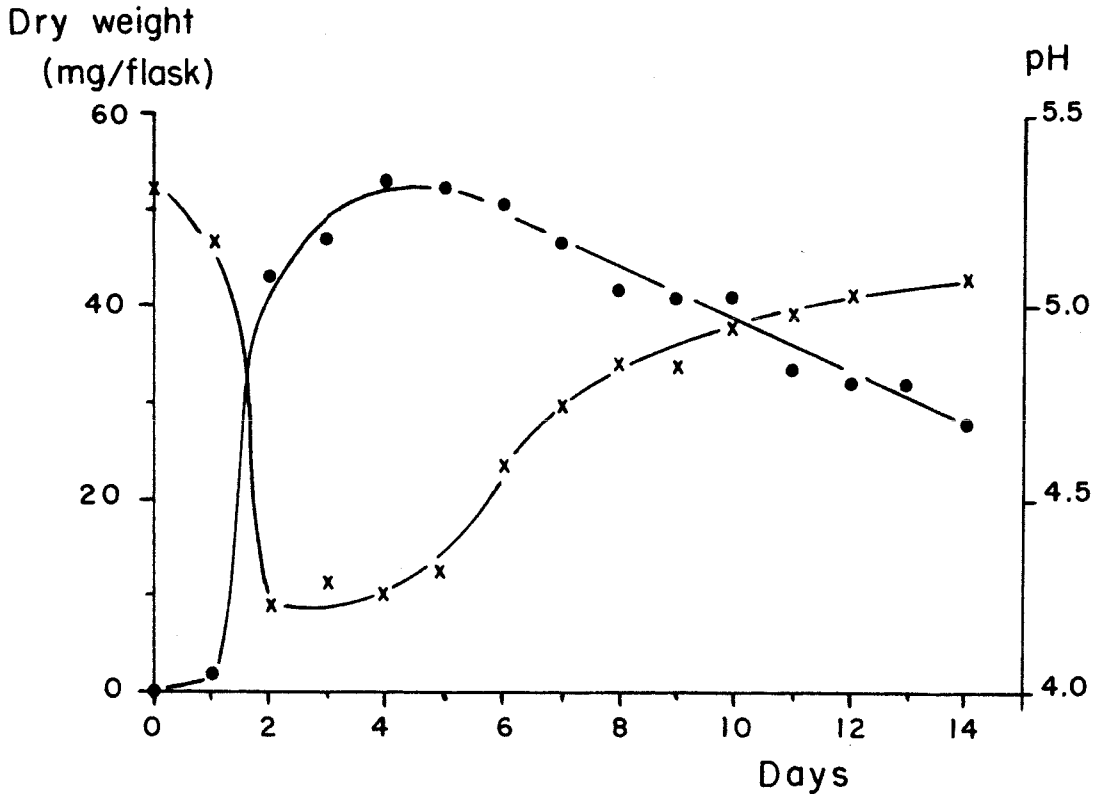

Figure 2. Growth of Neurospora crassa and the change of pH of the medium, at 35°C.

—●— Dry weight

—x— pH of the medium

Table 1. Production of laccase in wild strains of *Neurospora crassa*. (Substrate: DL-DOPA)

Strain	Duration of culture (days)					
	6	9	11	13	16	18
PR-12a	-	0	0	0	1	-
PR-18a	-	0	1	6	1	-
PR-16A	-	20	9	41	-	-
PR-20A	-	2	1	0	2	-
PR-15A	-	1	0	0	3	-
NA-1A	-	16	69	61	-	-
NA-2A	-	2	1	1	1	-
4A	38	57	88	118	-	-
854a	-	51	21	25	-	-
637A	-	3	1	3	22	-
5297a	1	5	-	-	6	2
4-121A	1	0	-	-	2	1
4-137a	1	0	-	-	3	1
1A	1	0	-	-	23	2
NS16-612	24	57	122	210	-	-

ammonium sulfate in the bottom of the beaker. The precipitate in the dialysis bag was centrifuged, dissolved in 5 ml of buffer, and dialyzed overnight against 500 ml of buffer.

The concentrated preparation was fractionated with ethanol at -10°C. The precipitate of the 50% to 60% ethanol fraction was dissolved in 5 ml of buffer, and dialyzed against 500 ml of buffer overnight. The preparation was further fractionated with ammonium sulfate at 0°C, the activity appearing in the 55%-70% saturation fraction. Twenty to fifty-fold purification of the enzyme was obtained. Protein determinations were made by measuring optical densities at 260 mμ and 280 mμ (10).

D. Substrate specificity and inhibitors

The substrate specificity of partially purified laccase is summarized in Table 2. These relative rates of color development of various substrates do not, however, represent stoichiometrically comparative values of reaction rates, because of the different molar absorbances of colored products. An extreme case is found in hydroquinone, which does not give any color in the presence of laccase and yet a rapid oxidation of it is detected by manometric measurements.

The spectrum of the substrate specificity agrees with reported laccases from the other sources; Japanese lacquer tree (5), Indo-Chinese lacquer tree (6), and mushrooms (11).

Table 2. Substrate specificity of Neurospora laccase, expressed by the relative rates of color development of reaction products, and by relative consumptions of oxygen in manometric measurements.

Substrates	Color of reaction products	Relative rate of color development	Relative uptake of oxygen
3,4-DL-dihydroxyphenylalanine	red	1.00	1.00
2,3-DL-dihydroxyphenylalanine	red	0.78	-
2,5-DL-dihydroxyphenylalanine	-	0.00	-
Pyrocatechol	yellow	2.40	1.83
Hydroquinone	-	0.01	2.12
Pyrogallol	yellow	0.99	-
Gallic acid	green	0.94	-
Guaiacol	orange	9.14	-
p-Phenylene diamine	purple	4.00	-
3,5-L-diiodotyrosine	orange	0.08	-
m-Tyrosine	-	0.00	-
o-Tyrosine	-	0.00	-
p-L-Tyrosine	-	0.00	-
p-Cresol	turbid	0.13	-
3-Hydroxyanthranilic acid	-	9.60	-
3-Hydroxykynurenine	yellow	2.40	-
Protocatechuic acid	yellow	1.56	-

Inhibition of Neurospora laccase by cyanide is shown in Table 3, and the effect of other inhibitors in Table 4.

E. Laccaseless strains

The strain, W913-83, sent from Dr. Westergaard, University of Copenhagen, is a morphological mutant which has a velvety appearance and is designated vt (also ty-1 in Part I of this thesis). This strain does not produce laccase nor tyrosinase. A cross was made between a vt strain, 31-224, and a good producer strain NA-1. Five complete asci were analyzed for laccase production and the results are shown in Table 5 with the production of other vt derivatives. There is no clear-cut one to one segregation regarding the production of laccase. It may be said that the amount of laccase is modified by more than one gene; nevertheless there is a correlation between the vt character and low production of laccase.

F. Immunological studies

Strain NS16-612 which produces the highest amount of laccase among the known strains in our laboratory and Strain NS16-614,* a laccaseless strain, were cultured for 13 days at 35°C in 160 flasks and 120 flasks respectively. The culture medium of each strain was collected and dialyzed against saturated ammonium sulfate solution in dialysis bags

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*See Table 5.

Table 3. Inhibition of laccase by cyanide.

Concentration of sodium cyanide	Percentage of inhibition
10^{-6}M	2.0
10^{-5}M	2.2
10^{-4}M	40.2
10^{-3}M	90.8
10^{-2}M	100

Table 4. Effect of inhibitors on laccase activity.

Compounds	Percentage of inhibition	
	10^{-3}M	10^{-2}M
Diethylthiourea	28.5 %	100 %
Sodium azide	84.5	99.6
Phenylthiourea	54.5	99.3
Sodium Cyanide	90.8	100
Carbon monoxide:	Bubbling CO through a laccase preparation in 0.1M phosphate buffer pH 6.0 for ten minutes did not inhibit the enzyme activity.	

Table 5. Laccase production of the progeny of the cross NS16 Na-1A[1] X 31-224a(vt),[2] and some other vt derivatives. The activity of laccase is expressed in c.u. per ml of the medium (12 day old culture) with DL-DOPA as the substrate.

		Morphological character	Laccase activity
ascus-1	NS16-111a	vt	3
	NS16-112a	vt	4
	NS16-113A	+	101
	NS16-114A	+	62
ascus-2	NS16-121A	+	71
	NS16-122A	vt	7
	NS16-123a	vt	3
	NS16-124a	+	160
ascus-3	NS16-211A	+	11
	NS16-212A	+	15
	NS16-213a	vt	2
	NS16-214a	vt	7
ascus-4	NS16-221A	+	53
	NS16-222A	vt	3
	NS16-223a	+	0
	NS16-224a	vt	4
ascus-5	NS16-611A	vt	3
	NS16-612A	+	149
	NS16-613a	vt	2
	NS16-614a	+	0
	NS56-13A	vt	320
	NS53-7a	vt	2
	NS53-12	vt	4

1) A strain sent from North Africa.

2) For the progeny of 31-224a see Fig. 7 of Part I of the present thesis.

overnight. The precipitates were centrifuged down, dissolved in 30 ml of 0.01M phosphate buffer (pH 6.0) and dialyzed against the same buffer overnight. The cloudy precipitates were centrifuged down and the supernatants were lyophilyzed. Light brown powder weighing 159 mg which had 650 c.u. (DOPA) per mg was obtained from NS16-612 medium and 67 mg of white powder without any enzymatic activity from NS16-614 medium. Five mg of each protein powder was injected into a rabbit three times a week for three weeks (total 45 mg). Twelve days after the last injection, 50 ml of blood was taken by cardial puncture from each rabbit and the serum was separated. The serum (0.5 ml) and a purified laccase solution (0.5 ml) were mixed and incubated for one hour at 30°C. The mixture was kept in the refrigerator overnight, centrifuged, and supernatant and precipitate were tested separately for residual activities colorimetrically using DL-DOPA as substrate (Owen et al., 14). The result is given in Table 6.

The anti-NS16-612 serum inactivates the enzyme about 90% and anti-NS16-614 serum does not inactivate it more than normal serum does. These results indicate the absence of cross reactive material with laccase in the culture medium of NS16-614 strain which produces little laccase.

An antiserum against tyrosinase had been prepared by Horowitz, Fling and Owen (12). A test for anti-laccase activity of this serum was made and no antibody activity was

Table 6. Antilaccase activity on purified laccase.

DL-DOPA was used as substrate.

	Normal serum		Anti NS16-612		Anti NS16-614		No serum	
	sup.	ppt.	sup.	ppt.	sup.	ppt.	sup.	ppt.
Exp. 1	102	0	13	16	107	4	128	--
Exp. 2	76	0	1	8	78	2	88	--

detected against purified laccase. Definite antibody activity of anti-NS16-614 serum against purified tyrosinase (60% inactivation of tyrosinase activity) was, however, detected. This fact was interpreted as indicating that the antiserum against the medium protein of NS16-612 contained anti-tyrosinase antibody mixed with anti-laccase antibody rather than a structural similarity of laccase and tyrosinase for the following reasons.

1. A partially purified tyrosinase preparation can absorb almost all anti-tyrosinase activity without affecting the anti-laccase activity of the serum.

2. The medium protein of NS16-612 does contain tyrosinase oxidizing activity, although purified preparations of laccase lack this activity.

3. Anti-tyrosinase antiserum does not inactivate laccase.

It may be concluded that there is no serological similarity between the structures of tyrosinase and laccase

G. Inducibility of laccase formation

In 1953 Fåhraeus and Lindeberg reported that various monophenols can increase laccase production of mushrooms, Polyporus zonatus and P. versicolor (13). Therefore, the inducibility of laccase in Neurospora crassa was investigated.

The mode of response of Neurospora strains to p-cresol (10^{-3}M) falls into four groups: non-inducible,[1] indifferent,[2] inhibitory,[3] and inducible.[4] From repeated experiments it was felt that although the mode of response is variable, in general the response pattern of each strain seems to be specific. Possible genetic control of inducibility was suspected and a cross between an inducible strain and an indifferent strain was made. The segregation of the character was, however, not clear-cut. The variation of laccase production from flask to flask was large.

H. Heat stability and paper electrophoresis

As the search for possible variant forms of laccase among different strains of Neurospora crassa was the main purpose of this investigation, the largest effort was made along this line. The results were, however, not promising. The following points will describe the facts.

1. Thermal inactivation curves of the both crude and purified enzyme are not, in most cases, of first order, although various kinds of buffer of different concentration and pH were tried.

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1) Laccase is not produced with or without the inducer.

2) Laccase is produced with or without the inducer.

3) Laccase production is inhibited by the inducer.

4) Laccase is induced by the inducer.

2. Heat stability changes greatly with purification; e.g. the half-life of 4A-laccase at 71°C with citrate phosphate buffer (pH 6.0) varies from 15 minutes (crude) to 8 minutes (16-fold purified), and that of 4-137 varies from 36 minutes to 10 minutes.

3. Paper electrophoresis using the Spinco Durrum type apparatus did not give sharp bands in spite of numerous trials in changing conditions.

4. None of the strains which have been collected in our laboratory gave marked differences in thermostability or electrophoretic migration.

III. DISCUSSION AND CONCLUSION

The "second phenol oxidase" of Neurospora crassa (1) was tentatively called laccase (2) on the basis of its substrate range. The present results give further support to the identification from both the substrate specificity and the spectrum of inhibitors.

External factors greatly influence the production of laccase; for example, temperature and concentration of sulfur and copper in the medium. Interstrain variations in amounts of the enzyme produced are very large, which suggests that more than one gene is responsible for the quantitative aspects of laccase formation.

The possibility that laccase might have a common structural origin with tyrosinase may be ruled out from the following facts. First, the genetic locus which controls the structural character of tyrosinase has no effect on the heat stability of laccase (2). Second, there is no serological cross reaction between the two enzymes. The amounts of tyrosinase and laccase produced by various wild strains of N. crassa are not correlated.

Inducibility of laccase in N. crassa seems to be strain specific. The considerable variability of the character, however, obscures the genetic variation.

Genetic variations in the structure of laccase were not revealed. Nevertheless, the author does not exclude

the possibility of existence of such a variation among the available strains in our laboratory. The failure to find them might be due either to the heterogeneity of the enzyme preparation or to the insufficiency in purification. Complexity of heat inactivation kinetics and spreading in paper electrophoresis may indicate heterogeneity. The long incubation (9-12 days) at relatively high temperature (35°C) may cause partial denaturations of the enzyme.

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