# A GENETIC STUDY OF TWO NEW STRUCTURAL FORMS OF TYROSINASE IN NEUROSPORA[1]

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IN previous work from this laboratory, three apparently unlinked genes concerned with the synthesis of tyrosinase in *Neurospora crassa* have been identified. These genes, designated *T*, *ty-1*, and *ty-2*, respectively, are not functionally equivalent. It was found that whereas the *T* locus has a structure-determining role in the synthesis of the enzyme, the genes *ty-1* and *ty-2* determine whether tyrosinase synthesis is constitutive or inducible but have no influence on the structure of the enzyme actually formed (HOROWITZ, FLING, MACLEOD, and SUEOKA 1960). The question arises whether any other genes of Neurospora, besides the *T* locus, participate in determining the structure of this enzyme. In the present paper, we report the results of a search for such genes.

Owing to the fact that tyrosinase is not essential for the growth of Neurospora, the usual selective procedures cannot be employed for detecting mutations affecting it. (The feasibility of developing a special technique for this purpose is under study.) Because of its nonessential character, however, the enzyme is presumably under weak selection in nature, and it seemed possible that it would show considerable structural variability in wild strains of Neurospora. Such variability had already been indicated by the fact that two different forms of the enzyme were found among a small number of standard wild type stocks in the California Institute of Technology collection (HOROWITZ and FLING 1953). We therefore undertook to examine the tyrosinase from an additional series of wild type strains, collected in various regions of the world. A group of 12 such strains, recently collected in nature, were obtained[3] and their tyrosinases compared with respect to their thermostability and rate of electrophoretic migration on paper. Based on these two criteria, four different forms of the enzyme could be distinguished among the strains. Two of these were indistinguishable from the previously known forms, and two were new types. The genetic relationship of the four forms has been investigated by crosses and heterocaryon studies.

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## MATERIALS AND METHODS

*Strains:* From material sent to the laboratory from various parts of the world, some 25 to 30 cultures of monilioid fungi were established. Purification from bacteria was accomplished by a few serial transfers of aerial hyphae. Each culture was crossed to protoperithecia of both mating types of wild type *Neurospora crassa*, and only those 12 which formed fertile crosses were used for further study. These were grown, and their tyrosinase was extracted, purified, and examined with respect to thermostability and electrophoretic migration. Several of the strains produced too little of the enzyme for characterization, and these were not studied further. Among the remainder, four different tyrosinases were found, as stated above, two of which were indistinguishable from types S and L, known from previous studies (HOROWITZ and FLING 1953), and two new types. The latter were designated Puerto Rico-15 (or PR-15) and Singapore-2 (or Sing-2), respectively, from the names of the strains in which they were discovered. The Puerto Rican strain was isolated from a sample of sugarcane bagasse provided by DR. TEAS and the Singapore strain from some dead bark sent by MR. THOMPSON. Both strains could be crossed with laboratory stocks of *N. crassa*, but fertility was not complete, as indicated by the fact that only random ascospores could be recovered from the crosses. Full fertility was attained by two or three generations of backcrossing to laboratory stocks, selecting for the backcrosses spores which carried one or the other of the new tyrosinase types. These crosses had the further effect of resolving whatever heterocaryotic mixtures may have been present in the natural strains. Evidence was obtained suggesting that the PR-15 strain was heterocaryotic for two different tyrosinases (see Discussion).

To avoid confusion, the designations PR-15 and Sing-2 will be retained for the derivative stocks obtained by backcrossing and for the characteristic forms of tyrosinase produced by them.

*Experimental procedures:* Crosses were carried out on synthetic medium (WESTERGAARD and MITCHELL 1947). Cultures for tyrosinase assay were grown at 25°C for four days on one of the low-sulfur minimal media described previously (HOROWITZ *et al.* 1960). Assays were performed colorimetrically at 30°C and *p*H 6, with 3,4-dihydroxy-DL-phenylalanine as substrate. Both crude and partially purified (HOROWITZ and FLING 1953) extracts were used as the source of enzyme. Thermal inactivation tests were done at 59° ± 0.02°C. For electrophoresis, a Spinco Model R Paper Electrophoresis Cell was used, with conditions as described in Table 1 and Figure 2. Further details of the electrophoretic procedure are given in an earlier publication (HOROWITZ *et al.* 1960).

## RESULTS

### *The four forms of tyrosinase*

*Thermostability:* Three different levels of thermostability were found among the four tyrosinases. Expressed as half-lives at 59°C, the thermostabilities are approximately five minutes for the L-form of the enzyme, 20 minutes for PR-15, and 70 minutes for the S and Sing-2 forms in 0.1 M sodium phosphate buffer at

*p*H 6. Thermal inactivation data are plotted in Figure 1. The previously reported value of three to four minutes for the L form (HOROWITZ and FLING 1953) was obtained in sodium-potassium phosphate buffer; the value is slightly higher in sodium phosphate (GEST and HOROWITZ 1958). The S and Sing-2 forms show very similar inactivation kinetics over the range 57°–67°C (SUEOKA 1959). We have recently isolated tyrosinase S in pure form (unpublished work from this laboratory), and have determined its thermostability. The half-life of the pure enzyme at 59° is about 65 minutes, in good agreement with the value obtained with partially purified preparations.

Thermostability measurements provide a convenient means for detecting structural differences between enzymes, but their usefulness is limited to cases where the thermostability difference is large, owing to the fact that the stability of proteins is strongly influenced by the composition of the medium. Thus, the rate of thermal inactivation of tyrosinase is affected by traces of substrate (HOROWITZ and FLING 1956), by the ionic environment (GEST and HOROWITZ 1958), and by the presence of other proteins in the solution. Impure preparations of the enzyme contain many substances capable of influencing its stability in one direction or the other, and the net effect of these substances will vary from one preparation to the next, depending on the composition of the initial, crude extract

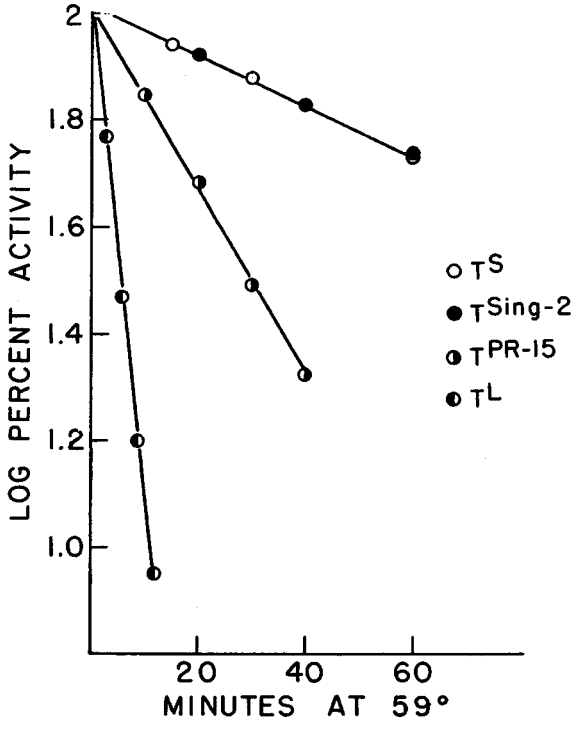


FIGURE 1.—Thermal inactivation of the four tyrosinases of *N. crassa* in 0.1 M sodium phosphate, *p*H 6.

and on the extent of purification. These effects can be minimized and often eliminated by carrying out thermal inactivation measurements with highly diluted samples of the enzyme preparation (HOROWITZ and FLING 1953), but some variability remains, particularly in the case of the most stable forms, S and Sing-2. Working with Sing-2, SUEOKA (1959) found occasional preparations whose half-lives at 59° varied by a factor of ±2 from the modal value of 70 minutes. It was thought at one time that a fifth form of tyrosinase, with a half-life of 83 minutes at 59°C, existed in our stocks (HOROWITZ and FLING 1957), but further work showed it to be indistinguishable from the known S-form by thermostability, electrophoresis, and genetic tests.

*Electrophoresis:* All four tyrosinases migrate toward the cathode in paper electrophoresis at *p*H 6. The L form is the fastest, S is intermediate, while Sing-2 and PR-15 are the slowest and are electrophoretically indistinguishable from each other. Figure 2 shows electrophoretic patterns of the individual enzymes and of two pairwise mixtures. All pairwise mixtures except Sing-2 + PR-15 can be separated electrophoretically; this indicates that the electrophoretically distinguishable types are structurally different. Table 1 summarizes the electrophoretic and thermostability characteristics which form the basis for recognition of the four enzyme types. The electromigration rates given in the table are observed rates, uncorrected for electroosmotic flow of the buffer.

Unlike the thermal inactivation rates, the rates of electrophoretic migration of the enzymes are, in our experience, independent of the purity of the preparations. The same values are found for crude extracts as for highly purified preparations. Occasionally, however, crude preparations of S and Sing-2 show one or two secondary bands of tyrosinase activity in addition to the principal

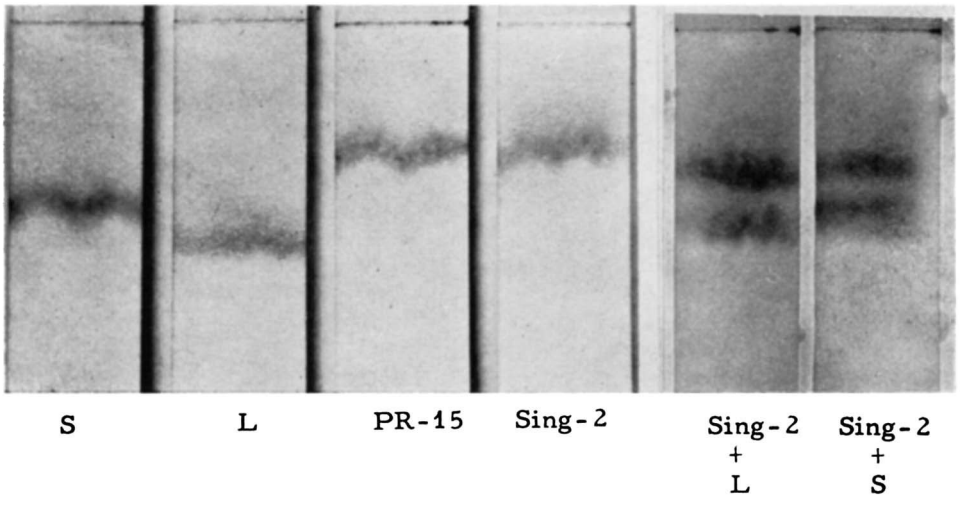


FIGURE 2.—Paper electrophoresis (20 hours) of the four tyrosinases and of two pairwise mixtures. The horizontal line at the top of each strip marks the point of application of the enzyme solutions. Migration is toward the cathode. Conditions as stated in Table 1. The enzymes were visualized by spraying the strips with a solution of 3,4-dihydroxy-DL-phenylalanine.

TABLE 1

*Properties of the four tyrosinase types*

| Type | Thermostability* (half-lives at 59°, min.) | Electrophoretic migration† (mm per hour, on paper) |
|---|---|---|
| S | 70 | 2.0 |
| L | 5 | 2.25 |
| PR-15 | 20 | 1.5 |
| Sing-2 | 70 | 1.5 |

* In 0.1 M sodium phosphate buffer, *p*H 6.
† In 0.05 M sodium phosphate buffer, *p*H 6, containing 0.1 percent bovine serum albumin. Current, 1.25 ma per paper strip in a Spinco Model R Paper Electrophoresis Cell. The rates are not corrected for electroosmotic flow of the buffer.

band. The secondary bands migrate more slowly than the principal band. The components giving rise to the extra bands are extremely labile. Storage of the preparations overnight in the refrigerator, freezing and thawing, or brief warming causes them to disappear. Apparently the same phenomenon has been observed by FOX, BURNETT and FUCHS (1959). These authors drew the conclusion that the labile components are normal precursors of tyrosinase and proposed that in the terminal stages of tyrosinase synthesis a thermolabile form of the enzyme (which they suggest may be identical with our L tyrosinase) is converted into a thermostable form (corresponding to our S). This interpretation is opposed by considerable evidence. In our experiments, the secondary bands do not migrate at the same rate as the L form of the enzyme, but slower. Furthermore, we have shown that tyrosinase S and L form stable mixtures both *in vitro* and *in vivo* (i.e , in mycelia heterocaryotic for the S and L alleles); if these mixtures are heated, the loss of tyrosinase activity precisely follows the course predicted for simple first-order inactivation of both forms (HOROWITZ and FLING 1956). A more reasonable interpretation of the secondary bands would seem to be that they result from interactions between the enzyme and other components of the crude extracts, or between tyrosinase molecules, giving rise to unstable associations. The subject clearly requires more study before conclusions can be drawn.

## *Genetics of the tyrosinases*

*Results of crosses:* Two series of crosses were carried out among the four strains. In Series A, the six possible nonreciprocal crosses were made, and complete tetrads were analyzed for segregation of tyrosinase types. In Series B, the crosses L × S, L × Sing-2, and L × PR-15 were made, and for reasons explained below, asci containing one unripe and three ripe spore pairs were selected for analysis. Cultures were grown from the ascospores, the enzyme was extracted and its thermostability and/or electrophoretic migration measured. In most cases, crude extracts could be used for these tests; partial purification of extracts was carried out where necessary to check results which appeared ambiguous or unusual.

Table 2 summarizes the results of the crosses. Simple Mendelian segregation of the enzymatic characters was observed in all cases. No recombination was detected. Recombination between the structurally dissimilar tyrosinases could be manifested in any of the following ways: (a) both recombinants might yield

TABLE 2

*Summary of genetic data on the tyrosinase types*

| Cross: X×Y Series | S×L* A† | S×L* B‡ | PR-15×L A§ | PR-15×L B‡ | Sing-2×L A§ | Sing-2×L B‡ | S×PR-15 A§ | S×Sing-2 A‡ | PR-15×Sing-2 A‖ | Totals |
|---|---|---|---|---|---|---|---|---|---|---|
| Complete tetrads | 73 | | 21 | | 30 | | 31 | 23 | 18 | 196 |
| M-I segregations | 31 | | 7 | | 8 | | 16 | 6 | 4 | 72 |
| M-II segregations | 42 | | 14 | | 22 | | 15 | 17 | 14 | 124 |
| Ratio X:Y | 146:146 | | 42:42 | | 60:60 | | 62:62 | 46:46 | 36:36 | |
| ¾-tetrads | 7 | 7 | 8 | 13 | 10 | | 16 | . . | . . | 61 |
| M-I segregations | 3 | 3 | 1 | 3 | 5 | | 5 | . . | . . | 20 |
| M-II segregations | 4 | 4 | 7 | 10 | 5 | | 11 | . . | . . | 41 |
| Ratio X:Y | 14:7 | 13:8 | 9:15 | 18:21 | 14:16 | | 29:19 | | | |
| ½-tetrads | 2 | 12 | 9 | 3 | 8 | | 2 | . . | . . | 36 |
| Ratio X:Y | 2:2 | 8:16 | 8:10 | 3:3 | 10:6 | | 3:1 | | | |
| ¼-tetrads | 2 | 5 | 4 | 5 | 3 | | 37 | . . | . . | 56 |
| Ratio X:Y | 2:0 | 3:2 | 4:0 | 2:3 | 2:1 | | 26:11 | | | |
| Total X:Y | 188:181 | | 86:94 | | 86:83 | | 120:93 | 46:46 | 36:36 | |
| Percent M-II segregations | 57.5 | | 73.8 | | 67.5 | | 55.3 | 73.9 | 77.8 | |
| Upper limit on recombination frequency | 0.014 | | 0.026 | | 0.030 | | 0.022 | 0.061 | 0.077 | |

* Includes previously published data on this cross (HOROWITZ and FLING 1953).
† Both thermostability and electrophoresis tests on 22 complete tetrads.
‡ Electrophoresis tests only.
§ Both thermostability and electrophoresis tests on all progeny.
‖ Thermostability tests only.

active new forms of the enzyme, (b) one recombinant might yield an active new form, the other an inactive form, (c) both recombinants might yield inactive forms. Since tyrosinase is presumably responsible for the melanization that accompanies the maturation of ascospores (SCHAEFFER 1953), tyrosinase-deficient recombinants might fail to mature and, if so, would not be detected. This would not be the case if melanization were not essential for ascospore germination, or if the tyrosinase of ascospores were entirely of maternal origin, but no conclusive evidence exists on either of these points. It is clear from these considerations that analysis of tetrads containing four ripe spore pairs can, in principle, detect outcome (a) with certainty, but (b) and (c) only doubtfully. Analysis of tetrads containing one unripe spore pair would detect (b), however, and this was the reason for the Series B crosses. As yet, there is no certain method for detecting (c). If recombination resulted in the abortion of one or both recombinant spore pairs, however, an increase in the frequency of incomplete asci over the number normally present would be observable. We have not made a statistical study of this point, but the frequency of unripe spores was not noticeably abnormal in these crosses.

*Calculation of upper limits on the recombination frequencies:* We wish to compute here the maximum recombination frequencies allowed by the data of Table 2. The lower limits are, of course, zero in all cases, since no recombinants were in fact found. The following discussion is based in part on a previous note (HOROWITZ 1953).

We shall consider first the crosses of Series B. If $d$ is the recombination frequency between two linked genes, the frequency of recombinant tetrads will be $2d$, since each such tetrad contains two recombinant and two nonrecombinant spore pairs. The frequency of nonrecombinant tetrads is $1 - 2d$. If $n$ tetrads are analyzed, the probability that none will show recombination is then $(1 - 2d)^n$. This expression is valid also for ¾-tetrads.

Assume that one recombinant type fails to mature and that a number of asci with one immature spore pair are analyzed, as in Series B. Owing to accidents of development, not all ascospores germinate, so that, in addition to ¾-tetrads, some ½- and ¼-tetrads will result. If the numbers of these are, respectively, $n'$, $p'$, and $q'$, then the probabilities $P'(0)$ that no recombination will be found are:

$$P'(0)_{3/4\text{-tetrads}} = (1 - 2d)^{n'}$$
$$P'(0)_{1/2\text{-tetrads}} = (1 - \tfrac{2}{3} \times 2d)^{p'}$$
$$P'(0)_{1/4\text{-tetrads}} = (1 - \tfrac{1}{3} \times 2d)^{q'}$$

and the probability that no recombinants will be found in the sample as a whole is

$$P'(0) = (1 - 2d)^{n'}(1 - 4/3d)^{p'}(1 - \tfrac{2}{3}d)^{q'}. \qquad (1)$$

By letting $d = 0.01$ in equation (1) and solving for $P'(0)$, the probability that no recombinants will be found if the segregating characters are one map unit apart is obtained. When this calculation is performed for the three crosses of Series B, it is found that the $P'(0)$'s are all less than four percent. (In applying the equation to Series B, cognizance is taken of the fact that ¾-tetrads of the type selected make up not over ten percent of all tetrads by multiplying the observed $n'$, $p'$, and $q'$ by 10). In view of the smallness of the chance that recombination would have been missed in these crosses, even assuming close linkage, it is unlikely that the ¾-tetrads contain a larger proportion of crossovers than other tetrads. The Series B tetrads can therefore be lumped with those of Series A for calculating upper limits on the recombination frequencies.

If both recombinants are viable and recognizable (outcome (a) above), then the probability $P(0)$ of no recombinants in a random sample of asci is, by reasoning analogous to that above,

$$P(0) = (1 - 2d)^n(1 - 5/3d)^p(1 - d)^q \qquad (2)$$

where $n$ = the number of complete + ¾-tetrads, $p$ = the number of ½-tetrads, and $q$ = the number of ¼-tetrads analyzed. If $P(0) = 0.05$, then $d$ defines a map interval within which the probability is 0.95 that one or more crossovers would have occurred in a sample of the given size. This value of $d$ is taken as the upper limit on the recombination frequency. In solving for $d$, the following approximation to equation (2) is useful where $p$ and $q$ are not equal to zero:

$$P(0) = e^{-2nd - 5/3pd - qd}. \qquad (2')$$

Upper limits obtained by equations (2) or (2') are listed in Table 2.

It is evident from the data of Table 2 that the thermostability and electrophoretic properties of the tyrosinase molecule are determined by a small chromosomal region. We refer to this region as the $T$ locus and, in the absence of contradictory evidence, assume that the four dissimilar tyrosinases represent four

alleles: $T^S$, $T^L$, $T^{PR\text{-}15}$, and $T^{Sing\text{-}2}$. The $T$ locus is in the right arm of linkage group I (HOROWITZ and FLING 1956).

*Heterocaryon experiments:* In a previous report, it was shown from the kinetics of thermal inactivation that the tyrosinase produced by a heterocaryon between $T^L$ and $T^S$ is a mixture of the L and S forms (HOROWITZ and FLING 1956). This conclusion has been confirmed in the present experiments by paper electrophoresis, by means of which the enzyme from the $(T^L)(T^S)$ heterocaryon can be separated into two zones corresponding to the L and S forms. When an extract of the heterocaryon was heated at 60°C for ten minutes before electrophoresis, only one zone of activity appeared on the paper strips, and this in the position known to be occupied by the S form.

Similar results have been obtained with heterocaryons $(T^L)(T^{Sing\text{-}2})$ and $(T^L)(T^{PR\text{-}15})$. As in the $(T^L)(T^S)$ case, these heterocaryons were balanced with the biochemical markers *hist-3* and *ad-3*. Again, mixtures of the two corresponding tyrosinases were formed by the heterocaryons, with no evidence of dominance or interaction. Thermal inactivation curves for the two new heterocaryons are shown in Figure 3. These results conform with what is expected on the assumption that the four tyrosinases are determined by a series of allelic genes.

## DISCUSSION

The most remarkable feature of the enzyme revealed by these studies is its

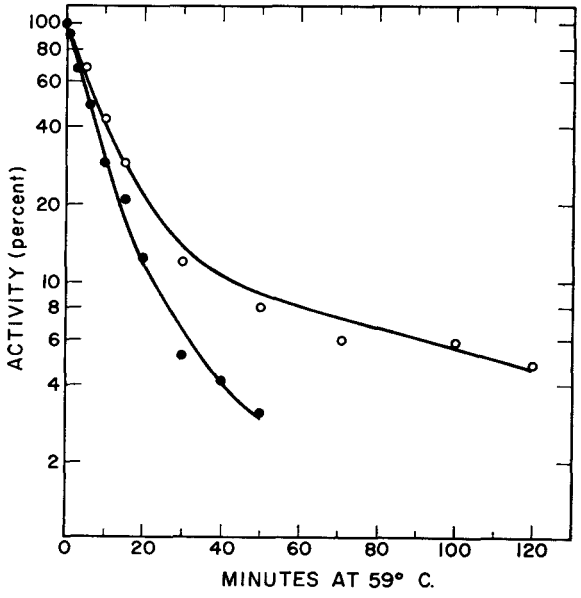


FIGURE 3.—Thermal inactivation of tyrosinases from heterocaryons. Open circles, Het (Sing-2) (L). Solid circles, Het (PR-15) (L). Conditions as in Figure 1. Points are experimental values; curves are theoretical for the following mixtures: open circles, 14% of half-life 75 minutes + 86% of half-life six minutes; solid circles, 15% of half-life 21 minutes + 85% of half-life five minutes.

high degree of structural variability in nature. This variability, or polymorphism, is so extensive that it is impossible to single out a wild type form of the enzyme. The four different forms were found in about a dozen wild strains of Neurospora by the use of two tests—electrophoresis and thermostability. It is reasonable to suppose that by applying other sensitive criteria, additional forms of tyrosinase could be found. Although the strains we have used originated for the most part in widely separated geographical regions, we have evidence for interclonal variation within a single region. The sample of sugarcane bagasse from which PR-15 was obtained also yielded an isolate which produced the S form. Judging from its thermal inactivation curve, the original PR-15 strain itself appears to have been a heterocaryon between $T^{PR\text{-}15}$ and $T^{L}$. The $T^{L}$ component was not recovered in the first generation progeny, however. This structural variability is possibly due to the weakness of the selection that operates on tyrosinase, a nonessential enzyme, in nature. No comparable study has been made on any essential enzyme of Neurospora, however.

Genetically, this study has found evidence for only one structure-determining genetic region for the enzyme. The fact that selective methods cannot be used for detecting recombinants among them limits the possibilities for subdivision of this region by further recombination studies on the four tyrosinases. The heterocaryon evidence, however, suggests that the differences observed among the four enzymes are determind by a functionally unitary region. It should be possible to attain further resolution of the *T* locus by the use of tyrosinase-deficient mutants. At the present time, two such mutants—*ty-1* and *ty-2*—are known, but neither of them is an allele of the *T* locus. These mutants have no effect on the structure of the enzyme, but control its induction (Horowitz *et al.* 1960).

It is instructive to compare our results with those of Markert (1950) on the genetics of tyrosinase in *Glomerella cingulata*. Markert studied a number of mutants in which the amount of tyrosinase activity ranged from a few percent of normal to ten or more times the normal activity, and he showed that these effects were produced by a number of different genes. It could not be determined from these results, however, which, if any, of the various genes had a structure-determining influence on the enzyme and which had merely quantitative effects. In a subsequent study, Markert and Owen (1954) attempted, by serological means, to detect structural alterations in the tyrosinase of the mutants, but without success. Our study agrees with the foregoing in indicating multigenic control of tyrosinase synthesis, but it clearly points to one locus as the structure-determining region.

The ease with which it is possible, by analysis of thermal inactivation curves, to obtain quantitative estimates of the amounts of two different tyrosinases in heterocaryons suggests that this system would be useful for investigating the effect of varying the nuclear ratio on the amounts of the two enzymes formed. Some data bearing on this question have been obtained (Sueoka 1959). They indicate a rather close parallelism between the nuclear ratio and the ratio of the

corresponding enzyme activities; but whether or not the different tyrosinases have the same specific or molar activities is not yet known.

SUMMARY

The tyrosinase of *Neurospora crassa* shows a remarkable degree of structural variability in nature. Four different forms of the enzyme have been found in a small number of newly collected wild strains. Two of these were indistinguishable from the previously known forms of the enzyme, S and L. The other two are new types, called Puerto Rico-15 (PR-15) and Singapore-2 (Sing-2). The four enzymes differ in their thermostability and/or electrophoretic properties. Simple Mendelian inheritance of the enzymatic differences was observed. No recombination was detected among the different forms, nor was dominance or interaction of any kind found in heterocaryons. It is concluded that the structural differences are determined by a small, functionally unitary genetic region, the *T* locus. The four tyrosinases appear to represent four different alleles at this locus.

ACKNOWLEDGMENT

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