GENETIC DETERMINATION OF TYROSINASE THERMOSTABILITY IN NEUROSPORA[1,2]

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IT was shown in a previous study that the tyrosinase activity of wild strain 4A of *Neurospora crassa* is influenced by the temperature of cultivation (HOROWITZ and SHEN 1952). The strain produces almost no tyrosinase activity when cultured at 35° C on a medium which favors the production of strong activity in 25° cultures. The evidence indicated that the temperature effect is not due to formation of a tyrosinase inhibitor, but to a net decrease in tyrosinase synthesis at the higher temperature. Attention has been called to the resemblance between this case and that of the Himalayan rabbit, in which the formation of melanin, the end-product of tyrosinase activity, fails to take place at body temperature (HOROWITZ 1951).

On the basis of the analogy with Himalayan, a search was made for strains in which tyrosinase is produced at both 25° and 35° (analogous with self-colored) and strains in which the enzyme is produced at neither temperature (analogous with albino). A number of strains analogous with self-colored, in the above sense, were found. When the tyrosinase in crude extracts of one of these strains was tested, it was found to be much more stable to heat than is the tyrosinase obtained from strain 4A. This discovery led us to an investigation of the following points: (a) the mode of inheritance of the thermostability, (b) the chemical basis of the difference in thermostability, and (c) the relationship between thermostability and enzyme production at 35°.

THE STRAINS

We distinguish no wild type of *N. crassa* with respect to tyrosinase thermostability, since among strains collected in nature it appears that some (referred to as T^S strains) produce the thermostable tyrosinase and others (T^L strains) the thermolabile tyrosinase. Of the original wild stocks obtained from various sources by BEADLE and TATUM (1945) and since maintained by serial transfer, one—Abbott 4A—is of the T^L type, and two others—12a and 25a—are T^S strains. Abbott 4A appears in the ancestry of all stocks which we have determined to be of the T^L type, and, similarly, the T^S character can be traced back to 12a and 25a. Neither character has ever been observed to arise by mutation in our stocks, but this does not exclude the possibility that they may do so.

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Strain 4A has been used as representative of the T^L type in many of the experiments to be described. Since asexually propagated lines of Neurospora may in time become heterocaryotic for heterogeneous assortments of genes through spontaneous mutation, we have repeated all essential tests with a recently isolated single-ascospore culture of type T^L, designated No. 4-137.

As representative of type T^S, strain No. 854a, containing both 12a and 25a in its ancestry, and a recent derivative of it, No. 4-121, were used. Inasmuch as strains of a given type gave identical results in all essential respects regardless of whether they were new isolates or old stocks, we shall dispense with the use of stock numbers in what follows and refer to the strains by the symbols T^S and T^L.

METHODS

The measurement of tyrosinase activity

Tyrosinase activity was determined colorimetrically and manometrically by the procedures previously described (HOROWITZ and SHEN 1952).

In the colorimetric method, the production of 2-carboxy-2,3-dihydroindole-5,6-quinone (hallachrome) is measured under standard conditions. This substance, a red pigment, is the first colored intermediate formed in the oxidation of tyrosine and 3,4-dihydroxyphenylalanine (dopa) by tyrosinase. It undergoes a base-catalyzed (non-enzymatic) decarboxylation and rearrangement, and subsequently an enzymatic oxidation, giving rise eventually to the precursor of melanin (RAPER 1932; MASON 1948). The decarboxylative rearrangement is the rate-limiting step in melanin formation (MASON and WRIGHT 1949), and at pH 6, where we have carried out all activity measurements, hallachrome is relatively stable (RAPER and WORMALL 1925). This property makes it possible to use the rate of hallachrome production as a measure of tyrosinase activity. It was shown by HOROWITZ and SHEN that the maximum rate of hallachrome production is proportional to the enzyme concentration in Neurospora extracts.

Because of its convenience, the colorimetric method is especially suitable for studies such as this one in which tyrosinase assays are carried out in large numbers. It has the additional advantage of good sensitivity (5 to 10 times more sensitive than the manometric assay), making it possible to test crude extracts at high dilutions and thereby minimize the possibility of interference by extraneous substances. In view of the general lack of uniformity among workers with regard to tyrosinase assay procedures, however, and because of the complexity of the tyrosine-tyrosinase reaction, we thought it desirable to check the colorimetric results by an independent method. For this purpose we have measured oxygen consumption manometrically in a number of experiments. The results obtained by the two methods are in good agreement, as shown in tables 1 and 4 below.

Cultures were grown for 4 or 5 days at 25° unless otherwise indicated, on the low sulfur liquid medium described by HOROWITZ and SHEN. The mycelium was washed with distilled water, weighed, and ground in a mortar with

sand and 20 ml of cold 0.1 M phosphate buffer, pH 6, per gram wet weight of mycelium. The resulting suspension was centrifuged to remove sand and debris. The supernatant, henceforth referred to as " crude extract," was used as a source of the enzyme in the early experiments. The more recent work has been carried out with a purified preparation to be described later.

Colorimetric determinations of tyrosinase activity were made with a Klett-Summerson photoelectric colorimeter, using filter No. 42. A measured quantity (0.5 ml or less) of the enzyme preparation was placed in a colorimeter tube, followed by sufficient 0.1 M phosphate, pH 6, to bring the volume to 4.5 ml. After temperature equilibration in a 30° water bath 0.5 ml of M/60 L-tyrosine or L-dopa (or M/30 DL-dopa) was added. Readings were taken at 5-minute intervals. Aeration was accomplished by shaking. Colorimetric rates as reported here are equal to the maximum increment in optical density (expressed as colorimeter units, *c.u.*) obtained in a 5-minute interval. With dopa as substrate for the purified enzyme, the maximum rate is attained in the first 5 minutes; with tyrosine there is a lag of up to 20 minutes, depending on the concentration of the enzyme. When the crude enzyme is used there may also be a brief lag with dopa.

Manometric measurements were made with the Warburg apparatus at 34.5°. Two ml of the enzyme solution were used in each vessel, with 0.25 ml of tyrosine or dopa solution in the sidearm, and alkali in the well. Manometric rates are given as the maximum oxygen consumption, in microliters, obtained in any 5-minute period.

It should be noted that dopa is stable at pH 6, so that the corrections for spontaneous oxidation which are necessary in experiments run at pH 7 or higher do not apply here.

Thermal inactivation

Most of the heat-inactivation experiments were carried out in the 54° to 60° temperature range, and for this purpose a thermostatically controlled water bath constant to $\pm$ 0.4° was used. The fluctuations in bath temperature are the source of the largest error in these experiments, since the temperature coefficient of inactivation is high in this temperature range.

Two different procedures have been employed in experiments where it was desired to measure the rate of thermal inactivation. In the first, 0.5-ml portions of the enzyme solution were measured into a series of 15-ml test tubes held in a rack. The tubes were stoppered, and at zero time the rack was immersed in the water bath. Tubes were removed at intervals and rapidly chilled in ice. The samples were diluted with buffer before assaying. 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, and a small volume of enzyme solution was run in at zero time. Samples were removed at intervals and chilled. The second method is the more accurate of the two, although both have given satisfactory results.

EXPERIMENTAL RESULTS

Thermostability of the enzymes

Table 1 shows the results of an experiment in which crude extracts of T^S and T^L were heated at 58° for varying periods of time and were then assayed for tyrosinase activity manometrically and colorimetrically, using dopa as substrate. The results obtained by the two assay methods are in good quantitative agreement and show that the tyrosinase activity from strain T^S is considerably more stable at 58° than is the activity from T^L, roughly 16 times more stable in this experiment.

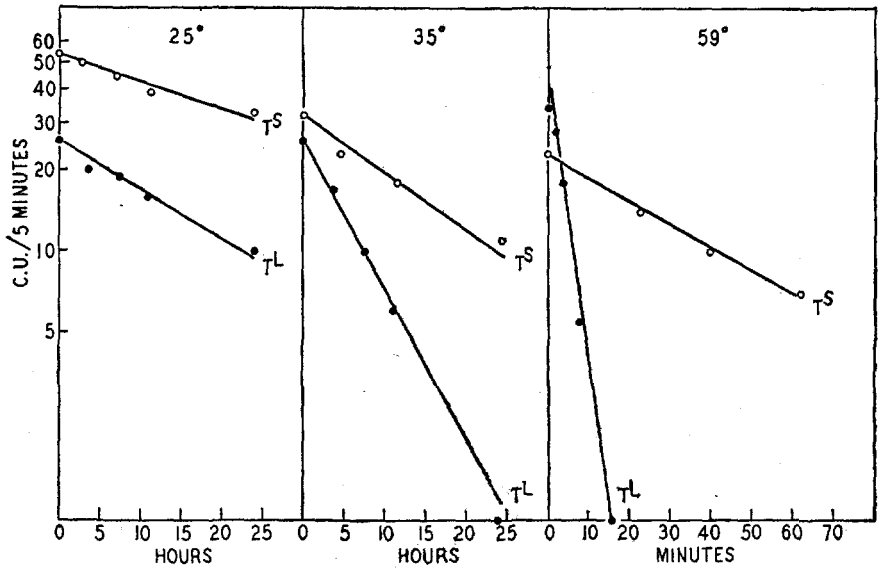

FIGURE 1.—Stability of T^S and T^L tyrosinases at three temperatures. Ordinate: residual activity in colorimeter units per 5 minutes, plotted on a logarithmic scale. Abscissae: time of exposure to the indicated temperature.

In figure 1 are plotted semilogarithmically the data from a series of experiments in which the stabilities of T^S and T^L tyrosinases were compared at three temperatures. The assays were colorimetric, with tyrosine as substrate. The data fall on a series of straight lines, within the limits of experimental error, indicating that the enzymes are inactivated in a first order process, i.e., a reaction whose rate is proportional to the tyrosinase concentration. The data of figure 1 can be summarized in terms of the half-lives of the enzymes at the given temperatures, as shown in table 2. Further consideration of the inactivation reaction will be postponed until after presentation of the genetical part of the investigation.

In other experiments, we have compared the T^S and T^L enzymes with respect to their pH optima and sensitivity to inhibition by cysteine. Although the possibility of small differences is not excluded by the experiments, no large

TABLE 1

Stability of T^S and T^L tyrosinases at 58°.

Each colorimeter tube contained 0.5 ml heat-treated crude extract + 4 ml buffer + 0.5 ml DL-dopa. Each Warburg vessel contained 1 ml heat-treated crude extract + 1 ml buffer + 0.25 ml L-dopa. c.u. = colorimeter units.

Strain	Minutes at 58°	Maximum rate			
		c.u. per 5 minutes	Percent	μl O_2 per 5 minutes	Percent
T^S	0	185	100	34.1	100
"	40	158	85	29.2	86
"	80	117	63	23.6	69
T^L	0	113	100	29.5	100
"	5	79	70	20.4	69
"	10	30	27	6.4	22
"	20	7	6.2	3.0	10

differences comparable to the thermostability difference were found. Both enzymes have optima in the neighborhood of pH 7 (with tyrosine as substrate), and both enzymes are completely inhibited by 2×10^{-4} M cysteine.

The genetics of tyrosinase stability

The inheritance of tyrosinase stability and lability was studied in the crosses $T^S \times T^L$, $T^S \times T^S$ and $T^L \times T^L$. Ascospores were isolated in order, and one member of each spore pair was tested for tyrosinase stability. Each spore isolate to be tested was cultured for 5 days at 25° on the low sulfur medium, and a crude extract was prepared from the mycelium. The extract was divided into

TABLE 2

Half-lives at three temperatures.

Temperature	Half-life (hours)	
	T^S	T^L
25°	29	16.5
35°	15.5	5.5
59°	0.5	0.05

two portions, one of which was incubated at 59° for 30 minutes while the other, serving as a control, remained at 0°. Both samples were then tested for tyrosinase activity, using tyrosine as substrate. The heat treatment was sufficient to destroy the labile enzyme, but not the stable enzyme. The results were thus essentially qualitative in nature and could be read by inspection. Nevertheless, colorimetric measurements were routinely made in order to make it possible to detect intermediate grades of stability. No clear intergrades were noted. The results are therefore presented in table 3 on a qualitative basis.

Three different crosses of $T^S \times T^L$ were made, involving different pairs of parents. A number of the progeny from one of the crosses showed very weak or no tyrosinase activity in the unheated control sample, and the thermosta-

bility could not be determined. Where this occurred the ascus is designated as incomplete. Similar negative progeny were obtained in some asci from the cross $T^S \times T^S$. A number of negative spores from the $T^S \times T^L$ cross were retested after having grown for 7 days instead of the standard 5, and they were then found to show some activity. The fact that negative spores occur in only certain crosses suggests that genes governing the time of development of tyrosinase activity may be segregating here.

TABLE 3

The inheritance of tyrosinase stability and lability.

S = good activity after 30 minutes at 59°. L = no activity after 30 minutes at 59°. − = no activity in unheated control.

Cross	Complete asci	Number	Incomplete asci	Number
$T^S \times T^L$	SSLL	3		
	LLSS	4		
	SLSL	4	None	
	LSLS	5		
	SLLS	2		
	LSSL	2		
$T^S \times T^L$	SSLL	5		
	LLSS	2		
	SLSL	1	None	
	LSLS	3		
	SLLS	0		
	LSSL	5		
$T^S \times T^L$	SSLL	4	SS-L	1
	LLSS	1	SSL-	1
	SLSL	1	L-SS	1
	LSLS	1	SLS-	1
	SLLS	2	S-LS	1
	LSSL	3	-SSL	2
	Total	48		7
	Grand Total	55, with 33 MII segregations.		
$T^S \times T^S$	SSSS	4	SSS-	2
			SS-S	1
			S-SS	1
			-SSS	2
			--SS	3
			-SS-	2
			---S	1
$T^L \times T^L$	LLLL	4	None	

A total of 48 complete and 7 incomplete asci were analyzed from the $T^S \times T^L$ crosses. All of the complete asci showed a 1 : 1 segregation for stability *vs.* lability, and the incomplete asci are all consistent with a 1 : 1 segregation. It thus appears that lability and stability are determined by a pair of alleles. The locus is at least 30 standard map units from its centromere (60 percent second-division segregations). Its linkage group is not known and may be difficult to determine, since the locus segregates almost at random with respect

to the centromere (complete randomness gives 67 percent second-division segregations).

It is proposed that the symbols T^S and T^L be used to designate these alleles, where T symbolizes *tyrosinase* and the superscripts designate *stable* and *labile*, respectively.

The chemical basis of stability and lability

We now take up the question of whether the difference in tyrosinase stability is based on a structural dissimilarity in the tyrosinases, or whether it can be accounted for on the basis of identical enzymes with differences in the production of a destructive or protective agent. It has already been shown that the inactivation is first order with respect to the enzyme, a fact which permits us to conclude only that the reaction is either monomolecular or pseudomonomolecular. By " pseudomonomolecular " reactions are meant bimolecular reactions which resemble a monomolecular process kinetically due to the fact that the concentration of one reactant does not change. The constant reactant can be a catalyst, or it can be a substance which is present in great excess. Since none of these possibilities has been excluded it is evident that the data up to this point do not give much information with respect to the mechanism of the inactivation. We have carried out three kinds of experiments in order to throw light on this problem: partial purification of the enzymes, dilution experiments, and mixing experiments. We shall consider these in order.

Purification of the enzymes.—A partial purification of the enzymes was effected by the procedure to be described. All operations were carried out at or near 0°. The course of the purification was followed by measuring activity of the fractions per unit of protein nitrogen. The latter was determined by micro-Kjeldahl digestion of 5 percent trichloroacetic acid precipitates, followed by Nesslerization.

Cultures were grown for 5 days at 25° in Fernbach flasks containing 200 ml of the low sulfur medium. A crude extract was prepared, using 3 ml of phosphate buffer per gram wet weight of mycelium. After centrifugation, the insoluble material was extracted again with the same volume of buffer and the two extracts pooled.

The pH was adjusted to pH 7 to 7.2 with 1 N NaOH, and manganous sulfate was added in the ratio of 1 ml of a 1 M solution per 20 ml of extract. The pH was brought back to pH 7.2 with NaOH and the precipitate removed by centrifugation. The precipitate was washed with a small volume of water and the washing added to the supernatant. This treatment removes the turbidity from the crude extract and leaves the tyrosinase activity in the clear supernatant.

Solid ammonium sulfate was added to the cold solution to 80 percent saturation (at 0°) and the precipitate, containing the enzyme, was centrifuged down and dissolved in 1 to 2 ml of water per gram of the original mycelium.

One volume of ice-cold acetone was slowly added to the aqueous solution containing the enzyme; the precipitate was centrifuged down and discarded.

More acetone was added to bring the concentration to 60 percent by volume, the precipitate was collected by centrifuging and was triturated with two successive small volumes (5 to 10 ml) of phosphate buffer (M/10, pH 6). The insoluble material was discarded.

The enzyme was reprecipitated from solution by the addition of 4 volumes of a saturated solution of ammonium sulfate, and it was again dissolved in a small volume of buffer. The clear, colorless solution was then dialyzed against phosphate buffer in the refrigerator.

The dialyzed solution constituted the partially purified enzyme preparation. In table 4 the activities of the crude and purified enzymes are compared. It can be seen that a 42- to 46-fold purification of the stable enzyme was obtained, but only a 12- to 14-fold purification of the labile enzyme, although identical fractionation procedures were employed in the two cases. The difference is attrib-

TABLE 4

Activity of crude and partially purified Neurospora tyrosinases.

Q_{O_2} = μl O_2/mg protein/hour. Protein obtained by multiplying the trichloroacetic acid precipitable nitrogen by the factor 6.25.

Strain	Preparation	Substrate	Activity		
			c.u./yN/5 min.	μl. O_2/yN/5 min.	Q_{O_2}
T^S	Crude	DL-dopa	1.94		
	"	L-tyrosine	0.99		
	Purified	DL-dopa	89.3		
	"	L-dopa		7.68	14,800
	"	L-tyrosine	41.8	7.43	13,700
T^L	Crude	DL-dopa	3.46		
	"	L-tyrosine	1.30		
	Purified	DL-dopa	40.0		
	"	L-dopa		3.20	6,140
	"	L-tyrosine	17.5	3.69	7,080

utable to a large loss in activity of the labile enzyme in the acetone step. The over-all recovery of the stable enzyme was 28 percent, and of the labile enzyme 7 percent.

Table 4 shows additionally that the two enzymes do not differ from one another significantly in their relative activities toward tyrosine and dopa. The two substrates are oxidized at nearly equal rates manometrically, but dopa is converted into hallachrome more than twice as fast as is tyrosine. This is consistent with the fact that an extra atom of oxygen is required in the oxidation of tyrosine. It can also be seen that no significant change in the relative reactivities of the two substrates resulted from the purification. This point is of some interest in view of the fact that several authors have noted an increase in diphenolase activity relative to monophenolase activity in the course of the purification of mushroom tyrosinase (KEILIN and MANN 1938; MALLETTE *et al.* 1948).

Finally, table 4 further illustrates two technical points mentioned previously: the proportionality between colorimetric and manometric rates when determined with the same substrate, and the greater sensitivity of the colorimetric method. The smallest detectable increment in volume of oxygen with the usual Warburg equipment (vessel constants around 1.5) is 0.75 μl, and the smallest detectable increment in optical density with the Klett-Summerson colorimeter is one colorimeter unit. But, as shown in the table, an amount of enzyme which in 5 minutes catalyzes the uptake by dopa of a quantity of oxygen equal to 10 times the minimal amount detectable, will in the same period of time produce a change in optical density equal to 90 times the minimal reading. With tyrosine as substrate the sensitivity difference is appreciably smaller.

Thermostability of the partially purified enzymes.—The stabilities of the partially purified enzymes were compared at 59°. The semi-log plots are shown in figure 2. The half-life of the T^L enzyme is 3 to 4 minutes, a value which does

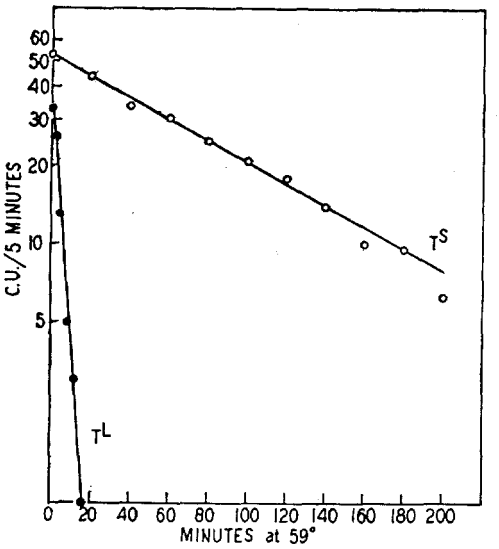

FIGURE 2.—Stability of the partially purified tyrosinases at 59°. Substrate: DL-dopa.

not differ from that obtained with the crude extract. The half-life of the purified T^S enzyme is approximately 70 minutes, a figure significantly greater than that found for the crude enzyme. It appears that purification of the tyrosinases accentuates rather than diminishes the difference in thermostability.

Dilution experiments.—In these experiments, enzyme stability was measured at various dilutions of the two enzymes. The interpretation rests on the following deductions from chemical kinetics: 1. If inactivation of the enzyme is monomolecular, the stability will be independent of concentration, and the fraction of the original activity remaining after time t will equal e^{-kt}, where k is a constant. 2. If the inactivation is pseudomonomolecular, the stability will increase with increasing dilution; the fraction remaining at time t will be given

by $e^{-k'tb}$, where b is the concentration of the second reactant (tyrosinase-destroying agent in this case). A special case is that in which the inactivation results from a reaction between the enzyme and the solvent. In this event, b will not change with dilution, and the reaction will therefore appear to be monomolecular by the dilution test. But since the solvent (phosphate buffer) is the same for both enzymes, this result will indicate a structural difference in the two enzymes, regardless of the actual mechanism of the inactivation.

In table 5 are shown the results of stability measurements made over a 32-fold range of enzyme concentrations. In each case, the indicated quantity of purified enzyme solution was diluted to the stated volume with buffer in a colorimeter tube. The tubes were heated in the water bath, cooled, and assayed with DL-dopa as substrate. The results show that dilution of the enzymes is without significant effect on their stability. If the reaction were pseudomono-

TABLE 5

Effect of dilution on the inactivation of T^S and T^L tyrosinases.

Strain	Ml enzyme	Final vol., ml	Inactivation temperature	Exposure time, minutes	Residual activity	
					c.u./5 min	Percent
T^S	0.5	4.5	59°	0	43	100
	2.8	"	"	40	151	63
	1.4	"	"	"	76.5	63
	0.7	"	"	"	33	55
	0.35	"	"	"	18.5	61
	0.175	"	"	"	8.5	57
	0.0875	"	"	"	4	53
T^L	0.2	2.25	54°	0	56	100
	0.8	"	"	8	123	55
	0.4	"	"	"	60	54
	0.2	"	"	"	29	52
	0.1	"	"	"	17	61
	0.05	"	"	"	9	64
	0.025	"	"	"	4.5	64

molecular, each fraction in the last column would approximate to the square root of the value above it, except in the special case discussed earlier. The essential constancy of the values is evidence for dissimilarity of the tyrosinases.

Mixing experiments.—The mixing experiment is based on the expectation that if the difference in tyrosinase stability is caused by differences in the enzymes themselves, then the rate of thermal inactivation of a mixture of the two will equal the sum of the individual rates; whereas if the difference is attributable to unequal concentrations of a destructive agent in the enzyme preparations, then the inactivation rate of a mixture will show an interaction effect.

Table 6 presents an experiment in which quantities of the two enzymes previously adjusted to approximately equal activities were inactivated singly and together at 59°. With the possible exception of one value, there is no significant departure from a simple additive effect. This experiment is thus in

accord with those already described in indicating a qualitative difference in the enzymes produced under the influence of the T^S and T^L alleles.

The second phenol oxidase

In the course of these studies a second phenol oxidase has been discovered in Neurospora. In preliminary experiments with crude preparations it has differed in the following respects from the enzymes described in this paper: it catalyzes a rapid oxidation of dopa, but has little if any effect on tyrosine; it is more thermostable than either of the tyrosinases we have been dealing with; it is produced in considerably larger amounts in 35° cultures than in the

TABLE 6

Inactivation of a mixture of T^S and T^L enzymes.

The indicated mixtures of enzymes and buffer were heated at 59°, then cooled and diluted to 4.5 ml with buffer, followed by 0.5 ml DL-dopa. Values in parentheses are the rates expected in the absence of interaction.

Ml enzyme		Ml buffer	Minutes at 59°	Activity c.u./5 min
T^S	T^L			
0.2	0	0.3	0	28
"	"	"	2	27
"	"	"	4	23
"	"	"	8	24
"	"	"	20	22
"	"	"	40	20
0	0.2	"	0	25
"	"	"	2	18
"	"	"	4	10
"	"	"	8	4
"	"	"	20	2
"	"	"	40	1
0.2	0.2	0.1	0	56 (53)
"	"	"	2	45 (45)
"	"	"	4	35 (33)
"	"	"	8	27 (28)
"	"	"	20	21 (24)
"	"	"	40	14 (21)

25° cultures; and it is found chiefly in the medium. Further studies are in progress.

On the basis of its known properties, it is safe to assert that the second phenol oxidase has had no significant disturbing effect in any of the experiments reported above.

Tyrosinase production at 35° in relation to thermostability

As mentioned previously, the existence of tyrosinases differing in thermostability was first discovered in two strains which differed also in their tyrosinase content at 35°. The findings were such as to suggest that the absence of tyrosinase from one of the strains when grown at 35° was directly related to the fact that it produced the thermolabile form of the enzyme. This inference

has not been supported by subsequent developments. The production of tyrosinase at 35° has not been inherited in a simple manner in the crosses so far analyzed. The segregations are not regularly 1 : 1, intermediate grades of expression are frequently obtained, and in several instances the initial classification of a culture has changed when the test was repeated at a later date. The results suggest multifactorial inheritance, with the possibility that uncontrolled physiological factors are important in the expression in some strains. All this is in marked contrast to the thermostability character. A number of attempts to demonstrate cytoplasmic inheritance of the ability to form tyrosinase at 35° have given only negative results.

It is not surprising to find that the enzyme content of a culture can vary independently of the thermostability of the enzyme. The net rate of enzyme production is of course determined by the rates of synthesis and loss. Thermal inactivation is but one of several processes which can contribute to the total rate of enzyme dissipation, and the extent to which thermal destruction influences the quantity of enzyme actually present in the cell cannot be predicted

TABLE 7

Phosphate-induced tyrosinase production at 35° in strain 4A.

The cultures were 6 days old. The heat treatment consisted of 30 minutes at 59°.

Phosphate concentration	Incubation temperature	Wet wt. of mycelium	c.u./0.1 ml extract/5 minutes			
			Untreated extract		Heat-treated extract	
			Tyr.	Dopa	Tyr.	Dopa
0.0074 M	25°	1.0 gm	18	19	0	1
"	35°	0.39 gm	0.1	2		1.4
0.3 M	35°	0.42 gm	60	106	0	12

without specifying the magnitude of the other parameters. Similar ideas have been embodied by DANNEEL (1941) in a hypothesis to account for the Himalayan color pattern in rabbits, and by ALLEN (1950) in a theory of thermophily in bacteria.

Experimental support of the above interpretation comes from the finding that tyrosinase production at 35° can be induced in strain 4A by nutritional means. Strain 4A is characterized by little or no tyrosinase production at 35° on the low sulfur medium, whereas at 25° it forms the thermolabile tyrosinase, as shown in this paper. When the medium is made 0.3 M in phosphate, however, 4A produces considerable activity at 35° (table 7). The enzyme is still of the thermolabile type, as shown in the table. (The thermostable fraction of the activity observed with dopa as substrate is due to the presence of a small amount of the second phenol oxidase.) Other experiments have shown that the half-life of the phosphate-induced enzyme at 59° is no different from that of the tyrosinase produced at 25° on unsupplemented medium.

It should be noted that owing to the high phosphate concentration which must be employed to obtain the above effect, the possibility remains open that the active substance is not phosphate, but a trace impurity.

DISCUSSION

Since all of the tests described above have failed to disclose the presence of any substance in the tyrosinase preparations capable of producing the observed difference in stability, we conclude that a structural dissimilarity, determined by the alleles T^S and T^L, exists between the two enzymes. This case thus provides direct evidence of the qualitative alteration of an enzyme as a result of gene substitution.

MAAS and DAVIS (1952) have recently presented evidence for the production of an abnormally thermolabile enzyme in a mutant of *E. coli* characterized by a requirement for pantothenic acid at temperatures above 30°. The enzyme concerned is that which couples β-alanine with pantoic acid to form pantothenic acid. This case appears to be similar to ours in principle, although it was not possible for the authors to carry out a genetic analysis in their strain of *E. coli,* a circumstance which is especially regrettable in view of the unusual origin of the temperature mutant (apparently spontaneously from a mutant requiring pantothenate at all temperatures).

In a series of investigations by PAULING, ITANO and collaborators (PAULING *et al.* 1949; ITANO and NEEL 1950; ITANO 1951) several electrophoretically abnormal forms of hemoglobin have been found in human patients. The abnormal hemoglobins appear to be inherited in a simple way, although only in the case of sickle-cell anemia have sufficient familial data been accumulated to make a definite genetic conclusion possible. The evidence shows that sickle-cell anemia is inherited as an incompletely recessive gene (NEEL 1949). Sickle-cell hemoglobin differs from normal in solubility as well as electrophoretically (PERUTZ and MITCHISON 1950).

Two alternative interpretations of these findings will be considered. The first of these, which we can call the template modification hypothesis, assumes that the specific molecular structure of each protein is copied from a template. Each template is considered to be associated with a particular gene, and gene mutation can result in a qualitatively new kind of template. The modified template will produce a correspondingly modified protein. This theory readily accounts for all of the observations, and it has much to recommend it, including the fact that it predicted the production of structurally altered proteins as a consequence of gene substitution.

The alternative, or rate modification hypothesis, assumes no particular mechanism of protein synthesis, but postulates that once synthesized, the protein undergoes secondary structural modifications which alter its stability, solubility, electrophoretic mobility, or other properties without destroying its essential specificity. These changes may result from limited hydrolysis, oxidation or the like, and are assumed to be controlled by certain genes. Mutation or substitution of one of these genes can, by blocking or accelerating one of the postulated reactions, cause the cell to accumulate a form of the protein which would otherwise be present only in traces. The model of this interpretation, as BONNER (1951) has pointed out, is provided by the metabolism of small

molecules, where it is known that mutations can result in the blocking of individual reactions and the accumulation of intermediates or their derivatives behind the block.

The rate modification hypothesis is not inconsistent with the template theory of protein synthesis, but it is important in template considerations because it provides an alternative explanation of findings which have been regarded as furnishing experimental support of the template idea. The immediate question is whether it is possible to design an experiment which would distinguish between the template modification and the rate modification hypotheses.

A desirable extension of the existing observations would be an investigation of the possibility of multiple allelism at the loci in question. If it can be shown that three or more alleles, each determining a structurally different protein, exist at a locus, the template modification hypothesis would be considerably strengthened, since it would seem most difficult to reconcile such a finding with the notion of mere quantitative increase in a normal cellular constituent. The fact that multiple allelism is known, or at any rate suspected, for a number of genes governing blood cell antigens leads one to expect the same situation will eventually be found among genes governing enzyme synthesis. The difficult question of pseudoallelism becomes critical at this point, however, and it is conceivable that an unambiguous genetic solution to the problem will not be possible by present methods.

SUMMARY

A pair of alleles, T^S and T^L, governing the thermostability of the enzyme tyrosinase, have been found in wild strains of *N. crassa*. Strains carrying T^S produce a tyrosinase characterized by a half-life of at least 30 minutes at 59°, whereas strains carrying T^L produce a tyrosinase with a half-life of 3 to 4 minutes at the same temperature. Allelism between T^S and T^L was inferred from an analysis of 55 asci from the cross $T^S \times T^L$. The locus is at least 30 standard map units from its centromere.

The following lines of evidence indicate that the difference in thermostability of the enzymes is based on a structural difference between them: 1. Partial purification of the enzymes does not cause them to become more alike in thermostability; on the contrary, the partially purified enzymes differ more than the crude enzymes. 2. Thermal inactivation of both enzymes occurs in a first order reaction; the slope of the first order inactivation curve does not change with dilution of the enzyme preparations. 3. The rate of thermal inactivation of a mixture of the two enzymes equals the sum of the individual rates.

The thermostability difference does not account for the fact that some strains of Neurospora produce tyrosinase when cultured at a high temperature (35°), whereas others do not.

A second phenol oxidase, differing in several properties from the tyrosinases, has been discovered in Neurospora.

The main results of the investigation are discussed in relation to the template theory of enzyme synthesis.

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