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A Comparison of the Properties of Two Forms of Tyrosinase from *Neurospora crassa*

ALFRED S. SUSSMAN[1, 2]

From the Department of Botany, University of Michigan, Ann Arbor, Michigan

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The differences between a thermostable (T^S) and thermolabile (T^L) form of tyrosinase from *Neurospora crassa* have been studied by means of a spectrophotometric technique using ascorbate or ferrocyanide. No significant differences in substrate specificity have been found in a survey of over 30 substances which included mono-, di-, tri-, and conjugated phenols. Catechol was found to be a substrate, in contrast to previous findings. Michaelis-Menten constants for the two forms of enzyme acting on L-tyrosine and L- and D-hydroxyphenylalanine (Dopa) also were found to be similar, as were the pH optima on L-Dopa and phenyl-4-catechol, and the response to several inhibitors. On the other hand, the activity of the thermolabile enzyme was lost more rapidly than that of the thermostable one after incubation for 5–15 min. in high concentrations of urea and formamide. These data are interpreted as support for the conclusion that the two forms of the enzyme differ mainly in their secondary and tertiary structure and not in their active centers.

INTRODUCTION

The template theory of gene action requires that the specificity inherent in a gene be reflected in an ordered arrangement of the amino acids in one protein, and in no other. These products of the gene, through their enzymic activities, determine the phenotype of the organism. Although this model is oversimplified, it has served as a useful framework for experimentation directed toward the understanding of the nature of the gene. Thus, one of the predictions that follows from the template theory is that different alleles should produce qualitative variations in gene products.

This question has been studied in detail in *Neurospora* wherein several forms of tyrosinase have been shown to exist (6). These were first distinguished on the basis of their heat resistance and, more recently, have been shown to differ in their electrophoretic properties (8, 9). On the other hand, in these earlier experiments with relatively impure preparations, these enzymes appeared to have similar substrate specificity (6), Michaelis-Menten constants, and pH optima (7). Therefore, on the basis of these experiments it was suggested that the different forms of tyrosinase were alike functionally but differed in structure. Among the three genetic loci which have been identified as influencing the production of tyrosinase, ty-1 and ty-2 regulate the production of the inducer, or of the repressor, whereas T-locus appears to determine the structure of this enzyme (8). The present work was initiated in order to explore the functional aspects of tyrosinase activity, using purified enzymes, so that a detailed comparison between the stable (T^S) and labile (T^L) forms could be made.

METHODS

Culture Techniques

Strains of *Neurospora crassa* which were used include 69-1113a (T^S), 65-811A (T^L), and 913_{83}A

[1] This work was performed during the author's tenure of a National Science Foundation Senior Postdoctoral Fellowship at the California Institute of Technology.

[2] Paper No. 1126 from the Department of Botany, University of Michigan.

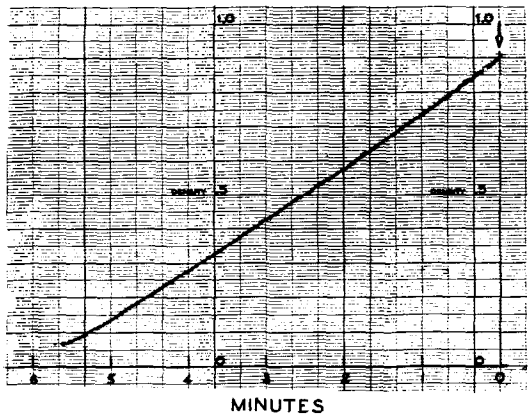

FIG. 1. Oxidation of 5.7×10^{-5} *M* L-Dopa by tyrosinase from *Neurospora* (T^S) as determined by the ascorbate assay. The abscissa of the recorder paper represents optical density and the arrow indicates the time of addition of the substrate.

(ty-1). Crude enzyme preparations were obtained by growing the organism on Vogel's medium N (21) containing only 6×10^{-5} *M* sulfate. Cultures were grown for 4 days at 25°C. in 125-ml. Erlenmeyer flasks containing 20 ml. medium.

Large quantities of mycelium were obtained through the use of aerated submerged cultures in 10-l. bottles containing 8 l. of the medium described above. The organism was grown in the dark at 25°C. for 3 days, upon which the mycelium was transferred under sterile conditions to another bottle containing 8 l. of 0.05 *M* phosphate buffer at pH 5.5 in which it was maintained for 2–3 days before harvest.

Crude extracts were prepared by grinding mycelium in 0.1 *M* phosphate buffer at pH 6.0 as described previously (6).

PREPARATION AND ASSAY OF ENZYMES

Purification was carried out through the stages described by these authors followed by passage through a Celite column.[3] By this means, the enzyme from T^L was brought to a specific activity of 157,500 units/mg. protein, which represents about a 400-fold increase in purity over the starting material. A unit of enzyme is defined as the increase in absorbancy with 2×10^{-2} *M* DL-dihydroxyphenylalanine (Dopa) as substrate in 0.1 *M* phosphate buffer at pH 6.0, at 25°C. for 5 min., using a Klett colorimeter with a blue filter (Klett #42). The purified enzyme from T^S which was used was a gift from Dr. Marguerite Fling and had a specific activity of 160,000. Recently, the enzyme from T^S has been crystallized[3] and shown to have a specific activity of 180,000 in this form. On this basis, the purified enzyme from T^L used was 80% pure and that from T^S was about 89% pure.

Comparisons between the manometric and colorimetric methods of determining the activity of tyrosinase from *Neurospora* have been made (6) thereby permitting the conversion of the specific activities given above into units which are equivalent to the amount of enzyme required to cause the uptake of 10 cu. mm. oxygen/min. (5). When expressed in these terms, each milliliter of the purified preparation from T^L had 1050 units of activity and that from T^S had 1155 units.

Unless otherwise stated, the following experiments were performed with the purified enzyme preparations.

Tyrosinase activity was determined by a method which is an elaboration of one first reported previously (2, 20, 23). This method measures the decrease in optical density of ascorbate at 265 mμ as a result of the oxidation of a substrate for the enzyme. Unless otherwise specified, the concentration of the reactants used in a total volume of 3 ml. was 7×10^{-6} *M* ascorbate, 1.5×10^{-5} *M* ethylenediaminetetraacetic acid (free acid), and 0.1 ml. enzyme to which 0.1 ml. of the substrate was added at zero time. All reagents were made up in 0.1 *M* phosphate buffer at pH 6.0, and the ascorbic acid and phenolic substrates were prepared immediately before use. The change in optical density was followed with a Cary recording spectrophotometer (model 11MS) running at a roller speed of 1 in./min., so that permanent records were available from which calculations could be made. Under these conditions, linearity was maintained with most substrates for at least 2 min., as can be seen in the sample curve in Fig. 1. Therefore, the decrease in optical density over the linear portion of the curve was computed, and the average rate per minute, corrected for the autoxidation of ascorbate, was used as the measure of tyrosinase activity. However, some reaction-inactivation was apparent almost immediately when catechol was used so that the values for its rate of oxidation are subject to considerable error. In the case of tyrosine, calculations were made from the linear part of the curve, after the lag period in its oxidation was over (Fig. 2).

The validity of this assay system for use with *Neurospora* was checked in several ways. Thus, the use of ascorbic acid, without substrates of tyrosinase, but with enzyme extracts, disclosed that there was no ascorbic acid oxidase present. That the concentration of ascorbic acid used was not critical in this system is revealed by the data in Table I in which no change in rate is shown. These data are in accord with the conclusions of others

[3] N. H. Horowitz and M. Fling, personal communication.

(11, 19) that ascorbate does not affect the rate of oxidation of catechol by polyphenol oxidase. That the rate of oxidation by tyrosinase in this system is directly proportional to enzyme concentration was also demonstrated so that this assay appears to be valid for use with diphenols.

On the other hand, increasing concentrations of ascorbate extend the lag in tyrosine oxidation, whether purified or crude enzyme preparations were used. There are additional reasons for suspecting the validity of the rates obtained with monophenols, a detailed discussion of which will be deferred until later.

A test of the specificity of the assay was performed by using extracts of strains of *Neurospora crassa* which were devoid of tyrosinase activity. Extracts of ty-1 and of an unnumbered strain which I had isolated induced no oxidation of ascorbate above the background of autoxidation in the presence of 3.3×10^{-5} *M* L-Dopa, thereby strengthening the assumption that tyrosinase activity was being measured by this technique.

Ferrocyanide was used as a reductant in the spectrophotometric assay of tyrosinase by substituting 0.02 *M* $K_4Fe(CN)_6.3H_2O$ for the ascorbate in the system described above. The rate of increase in optical density at 420 mμ upon the oxidation of the ferrocyanide was used as the index of activity. No effect of ferrocyanide concentration upon the rate of oxidation of 6.7×10^{-5} *M* L-Dopa was detectable between 0.0067 and 0.033 *M*. Furthermore, ferrocyanide, in the presence of substrate, was oxidized in direct proportion to enzyme concentration so that the method proved to be feasible. Several advantages accrue to the use of ferrocyanide instead of ascorbate, including:

1. The assay starts from zero optical density so that background "chatter" in the instrument is minimized.

2. Ferrocyanide is not as sensitive as ascorbate to changes in pH, especially in the acid range, because its oxidation-reduction potential is less affected.

3. Ferrocyanide is stable in solution and can be stored for repeated use.

4. Tyrosinase activity in the presence of substrates, as well as inhibitors, which absorb strongly in the region of 265 mμ can be assayed, whereas in the ascorbate system it is difficult to do so.

On the other hand, the sensitivity of the ferrocyanide technique is less than half that of the one using ascorbate, on the basis of comparisons using the same concentration of substrate in both assays.

Specific activities during enzyme purification were computed on the basis of protein determinations which were performed by the method of Lowry *et al.* (16).

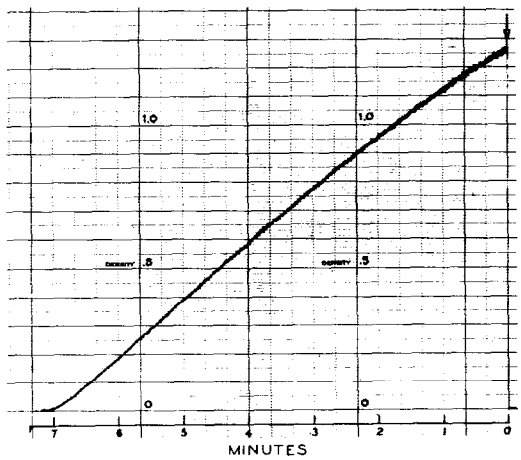

FIG. 2. Oxidation of 7.1×10^{-5} *M* DL-tyrosine by tyrosinase from *Neurospora* (T^s) as determined by the ascorbate assay. Note the "lag" period which extends over the first 4 min. The abscissa represents optical density and the arrow indicates the time of addition of the substrate.

TABLE I

EFFECT OF VARYING THE CONCENTRATION OF ASCORBATE IN THE ASSAY OF *NEUROSPORA* TYROSINASE

A crude enzyme preparation was used.

Ascorbic acid concentration	Δ opt. dens. × 10³/min.
Substrate:	
3.3×10^{-5} *M* DL-Dopa	
3.5×10^{-6}	114
7.0×10^{-6}	122
1.0×10^{-5}	117
1.4×10^{-5}	114
2.1×10^{-5}	125
Substrate:	
6.7×10^{-7} *M* L-tyrosine	
6.7×10^{-6}	17
9.0×10^{-6}	18
1.1×10^{-5}	17

RESULTS

Michaelis constants were derived as a means of comparing the enzymes from T^s and T^L. This was accomplished for L-tyrosine, L-Dopa, and D-Dopa, with both crude and purified enzymes, with the results shown in Table II and Fig. 3. In general, there appears to be a close similarity between the K_S values of the two forms of tyrosinase.

TABLE II

MICHAELIS CONSTANTS (K_S) FOR STABLE (T^S) AND LABILE (T^L) FORMS OF TYROSINASE FROM *NEUROSPORA*

Data calculated by least-squares analysis of at least eight points. Paired values were obtained from experiments performed on the same day.

Substrate	Purity of enzyme	K_S of tyrosinase from:	
		T^S	T^L
L-Tyrosine	Crude	1.8×10^{-4}	—
L-Dopa	Crude	1.9×10^{-4}; 5.3×10^{-4}	—
L-Tyrosine	Purified	5.3×10^{-5}	1.0×10^{-4}
L-Dopa	Purified	1.9×10^{-4}; 2.6×10^{-4}	4.0×10^{-4}; 5.3×10^{-4}
D-Dopa	Purified	1.4×10^{-4}; 1.5×10^{-4}	1.2×10^{-4}; 2.0×10^{-4}

Their substrate specificity was also studied, and the results are summarized in Table III. Once again there is a close parallel between the activities of the two enzymes. These data also extend the range of substrates utilized by *Neurospora* tyrosinase to include catechol, phenyl-4-catechol, caffeic acid, chlorogenic acid, 4-hydroxyphenol acetic acid, pyrogallol, 3,4-dihydroxybenzoic acid, and 3,4-dihydroxybenzaldehyde, as well as the ones reported previously (6, 10). It is noteworthy that phenyl-4-catechol is the best substrate of those tried, and catechol is rapidly oxidized by the enzyme, contrary to previous reports (10, 15).

Because measurement of tyrosinase activity on catechol by means of the ascorbate assay was rendered inaccurate by the high absorbance of the substrate at 265 mμ, the ferrocyanide assay was applied. In addition, the color of oxidized catechol was followed directly at 460 mμ in order to determine enzyme activity by another means. Both methods of assay, according to the data in Table IV, establish that catechol is a substrate for *Neurospora* tyrosinase. However, a comparison of the curves in Fig. 4 with those in Figs. 1 and 2 will reveal that reaction–inactivation of the enzyme is extremely rapid in the presence of catechol as compared with that when other substrates are used. In fact, the oxidation of this substrate might well be overlooked in assays like the manometric ones in which changes within the first minute of mixing are difficult to measure.

The pH optima of the two forms of tyrosinase were determined for L-Dopa and phenyl-4-catechol. As the curves in Figs. 5 and 6 show, the optimum is broad and extends from pH 5 through 8 for L-Dopa and from pH 5 through 9 for phenyl-4-catechol. Some difficulties in measurement were experienced above pH 9 due to the toxicity of borate and the high rate of autoxidation of the phenolic substrates and ascorbate. In addition, the enzyme has been found to be unstable above pH 10.

Another difficulty associated with the use of ascorbate is the increase in its oxidation–reduction potential as the pH is lowered (1). Therefore, the measurement of tyrosinase activity at pH 4.0 and below might be handicapped by the decreased oxidizability of ascorbate. This was checked by the use of the ferrocyanide method, as a result of which the data in Table V were obtained. These results substantiate those presented in Fig. 5, placing the pH minimum at about 3.5 for L-Dopa. However, it should be noted that the enzyme may be irreversibly inactivated below pH 4.5 so

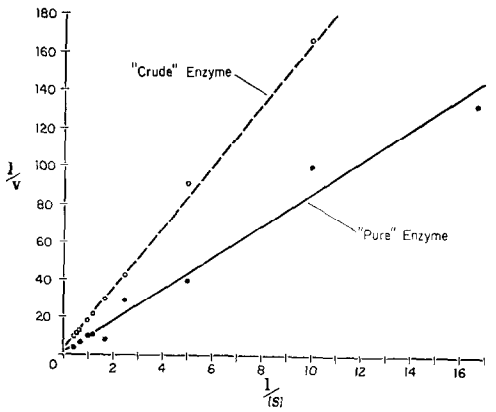

FIG. 3. Lineweaver-Burk plot of activity of tyrosinase (T^S) from *Neurospora*, using "purified" and crude preparations. Substrate used was L-Dopa. Units used: V = △ optical density/min.; S = molarity of substrate.

TABLE III

VELOCITY OF OXIDATION OF VARIOUS PHENOLS IN THE PRESENCE OF THE STABLE (T^S) AND LABILE (T^L) FORMS OF TYROSINASE

Substrate	Concentration	Velocity[a] × 10^3		Per cent activity on L-Dopa	
		T^S	T^L	T^S	T^L
	M				
L-Dihydroxyphenylalanine (L-Dopa)	3.6×10^{-5}	72.5	68.0	100	100
Catechol	0.7×10^{-5}	24.5	22.0	33.8	32.4
	3.6×10^{-5}	77.5	80.0	107	117
	2.1×10^{-3}	0	0	0	0
Resorcinol	3.6×10^{-4}	1.0	2.0	1.4	2.9
Orcinol	3.6×10^{-3}	0.4	2.2	0.5	3.2
Hydroquinone	3.6×10^{-5}	2.0	2.0	2.8	2.9
	7.1×10^{-5}	2.5	1.5	3.4	2.2
	3.6×10^{-4}	5.6	2.1	7.7	3.1
2-Methylhydroquinone	3.6×10^{-5}	0	0.6	0	0.9
Phenyl-4-catechol	3.6×10^{-5}	303.5	⊖	418	—
	0.7×10^{-5}	86.0	83.0	118.6	122.0
3,4-Dihydrocinnamic acid (caffeic acid)	7.1×10^{-5}	61.8	60.5	85.2	88.9
Chlorogenic acid	7.1×10^{-5}	63.2	67.2	87.2	98.6
p-Phenylenediamine	3.6×10^{-4}	2.0	2.0	2.8	2.9
L-Tyrosine	3.6×10^{-5}	43.1	38.4	59.5	56.4
DL-*m*-Tyrosine	7.1×10^{-5}	0.6	4.0	0.9	5.9
DL-*o*-Tyrosine	7.1×10^{-5}	1.0	3.0	1.4	4.4
3-Iodotyrosine	3.6×10^{-5}	3.9	4.4	5.4	6.5
3,5-Diiodotyrosine	3.6×10^{-5}	3.0	6.0	4.1	8.8
3-Nitrotyrosine	7.1×10^{-5}	1.3	5.2	1.8	7.6
	3.6×10^{-5}	0.7	1.6	0.9	2.3
Phenol	1×10^{-4}	16.3	12.6	22.5	18.5
	4.2×10^{-4}	41.8	36.2	57.7	53.2
4-Hydroxyphenol acetic acid	7.1×10^{-5}	6.8	9.3	9.4	13.7
	3.6×10^{-4}	35.1	30.6	48.4	45.0
2,4,6-Trichlorophenol	7.1×10^{-5}	1.9	3.7	2.6	5.8
2-Aminophenol	3.6×10^{-5}	5.4	4.4	7.5	6.5
α-Naphthol	7.1×10^{-5}	1.2	0.8	1.7	1.2
Pyrogallol	7.1×10^{-5}	9.2	6.0	12.7	8.8
	3.6×10^{-4}	54.9	42.1	75.7	61.9
Gallic acid	1.8×10^{-4}	0.7	1.1	1.0	1.6
Shikimic acid	3.2×10^{-5}	0	2.9	0	4.3
Quinic acid	3.6×10^{-3}	0	0	0	0
Phloroglucinol	3.6×10^{-5}	0.6	1.4	0.8	2.1
3-Hydroxybenzoic acid	7.1×10^{-5}	1.0	1.5	1.4	2.2
	3.6×10^{-4}	1.1	1.8	1.5	2.6
4-Hydroxybenzoic acid	7.1×10^{-5}	3.3	0	4.6	0
3,4-Dihydroxybenzoic acid	7.1×10^{-5}	11.7	11.1	16.1	16.3
	3.6×10^{-4}	44.2	36.3	61.2	53.4
2,3-Dihydroxybenzoic acid	7.1×10^{-5}	1.7	2.3	2.3	3.4
	3.6×10^{-4}	1.1	1.3	1.5	1.9
3,4-Dihydroxybenzaldehyde	3.6×10^{-5}	9.3	7.6	12.8	11.2
	7.1×10^{-5}	11.7	14.1	16.1	20.7
4-Hydroxybenzaldehyde	7.1×10^{-5}	0.3	0.9	0.4	1.3

[a] Velocity calculated as Δ optical density of ascorbate/min. with 0.1 ml. enzyme, corrected for differences in activity of the enzyme by using the rates for 3.6×10^{-5} *M* L-Dopa as standards. The autoxidation of substrates is subtracted in the values given above.

that activities measured below this point are difficult to interpret.[3]

The effect of metal-binding agents and some other compounds is shown in Table VI. As expected, phenylthiourea, sodium diethyldithiocarbamate, cysteine, and sodium azide are extremely inhibitory, whereas benzoic acid is somewhat less so. On the other hand, quinic and shikimic acids, intermediates in aromatic biosynthesis, do not inhibit the enzyme, even in relatively high concentrations. No difference in the action of these substances on the two forms of the enzyme was detectable.

TABLE IV

OXIDATION OF CATECHOL BY *NEUROSPORA* TYROSINASE MEASURED BY MEANS OF THE FERROCYANIDE ASSAY AND DIRECTLY BY THE CHANGE IN OPTICAL DENSITY AT 460 mμ

A purified enzyme from T^S was used throughout.

Catechol concentration	Assay method	Δ opt.density × 10^3
M		
3.3×10^{-5}	"Direct"	10.0
6.7×10^{-5}		19.0
1.7×10^{-4}		43.0
3.3×10^{-4}		47.5
1.0×10^{-3}		24.5
1.7×10^{-3}		14.5
6.6×10^{-5}	Ferrocyanide	46.0
1.7×10^{-4}		81.5
3.3×10^{-4}		82.0
6.7×10^{-4}		85.5
1.0×10^{-3}		91.5
1.3×10^{-3}		44.0
1.7×10^{-3}		19.0
1.0×10^{-2}		20.0

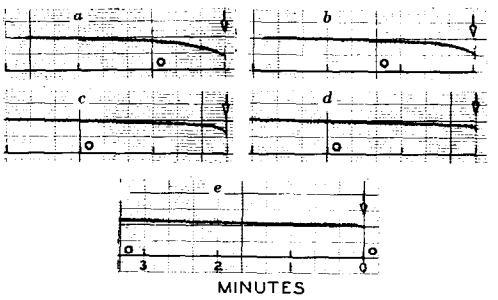

FIG. 4. Oxidation of catechol by tyrosinase from *Neurospora* (T^S) as determined in the ferrocyanide assay. Scale and axes as in Figs. 1 and 2. Concentrations of catechol used are as follows: curve *a*, 1.8×10^{-4} *M*; curve *b*, 3.6×10^{-4} *M*; curve *c*, 7.1×10^{-4} *M*; curve *d*, 1.4×10^{-3} *M*; curve *e*, 1.8×10^{-3} *M*.

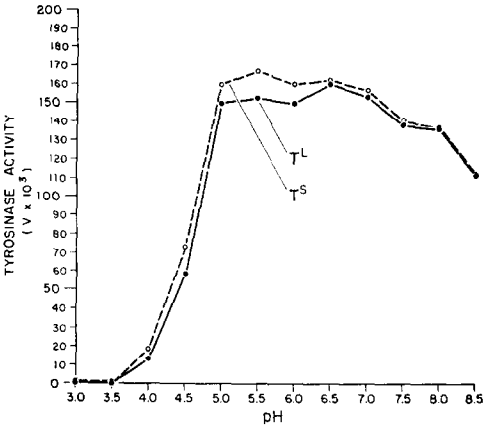

FIG. 5. Effect of pH upon the activity of tyrosinase from *Neurospora* on L-Dopa. Closed circles represent the values obtained for the thermolabile enzyme (T^L) and open circles those from the thermostable enzyme (T^S). The buffers were used at a concentration of 0.1 *M* and included the following: citrate–phosphate (pH 3.0 through 7.0), phosphate (pH 7.5 and 8.0), and Tris (pH 8.0 and 8.5).

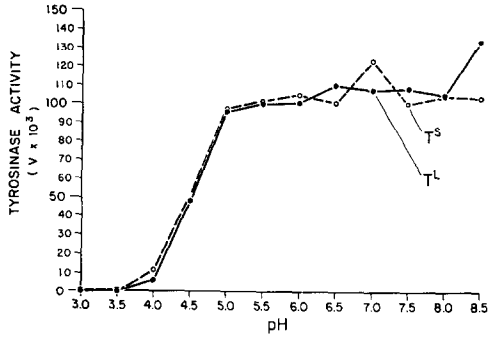

FIG. 6. Effect of pH upon the activity of tyrosinase from *Neurospora* on phenyl-4-catechol. Closed circles represent the values obtained for the thermolabile enzyme (T^L) and open circles those from the thermostable enzyme (T^S). Buffers used as in Fig. 5.

TREATMENT WITH UNFOLDING AGENTS

Substances which induce the unfolding of proteins (22) were added to the enzyme and

incubated for up to 15 min. before the activity was measured. The appropriate amount of urea was added to 0.1 ml. of the enzyme and incubated. At the end of this time the volume was made up to 3 ml., and the assay was carried out as before. Table VII discloses that urea affects the two forms of tyrosinase differentially in that the thermolabile form is more easily inactivated than is the thermostable one. Essentially the same results were obtained when formamide was used in the same way as urea, as the data in Table VIII reveal. In the case of both substances the difference in the response of the two enzymes did not appear until after an incubation period of at least 5 min., even though inhibition was observable immediately. The inactivation of both enzymes that occurred on standing, in the absence of urea or formamide, has been observed with purified enzymes by others[3] and appears to be characteristic of tyrosinase from *Neurospora*.

TABLE V

EFFECT OF pH ON THE STABLE (T^S) AND LABILE (T^L) FORMS OF TYROSINASE IN *NEUROSPORA*, AS MEASURED BY THE FERROCYANIDE TECHNIQUE

Concentration of L-Dopa used as substrate was 7.2×10^{-4} *M*. Data corrected for autoxidation.

pH	Buffer	Δ opt. dens. × 10^3		Per cent control rate	
		T^S	T^L	T^S	T^L
6.0	Phosphate	186	160	100	100
3.0	Citrate	0	0	0	0
3.5	Citrate	4	7	2	4
4.0	Citrate	39	25	21	16
4.5	Citrate	121	110	65	69
5.0	Citrate	194	180	104	112
9.0	Tris	69	57	37	36

TABLE VI

EFFECT OF METAL-BINDING AGENTS AND OTHER COMPOUNDS UPON THE STABLE (T^S) AND LABILE (T^L) FORMS OF TYROSINASE FROM *NEUROSPORA*

Concentration of L-Dopa used as substrate was 3.6×10^{-5} *M*.

Substance	Concentration	Δ opt. dens. × 10^3		Per cent control rate	
		T^S	T^L	T^S	T^T
	M				
Control	—	148	142	100	100
Phenylthiourea	2.2×10^{-5}	52	—	35	—
	1.1×10^{-4}	28	24	18	17
Sodium azide	7.1×10^{-5}	10	11	7	8
Cysteine	3.6×10^{-5}	75	66	50	47
	1.8×10^{-4}	29	21	19	15
Benzoic acid	7.1×10^{-4}	49	48	32	34
Sodium diethyldithiocarbamate	7.1×10^{-5}	14	9	10	7
Control[a]	—	155	115	100	100
Quinic acid[a]	3.6×10^{-3}	151	117	97	101
Shikimic acid[a]	3.6×10^{-3}	142	111	91	96

[a] Analyzed by the ferrocyanide method.

TABLE VII

EFFECT OF UREA UPON THE STABLE (T^S) AND LABILE (T^L) FORMS OF TYROSINASE FROM *NEUROSPORA*

The substrate was 7.1×10^{-5} *M* L-Dopa.

Urea concentration	Minutes in urea	Δ opt. dens. × 10^3		Per cent control velocity	
		T^S	T^L	T^S	T^L
M					
0	—	150	116	100	100
8	0[a]	123	92	82	79
12	0[a]	82	63	54	54
14.4	0[a]	45	33	30	29
0	5[b]	137[c]	99[c]	100	100
12	5	66	38	48	38
0	15[b]	114	65	100	100
12	15	66	19	58	29
14.4	15	21	6	18	9

[a] The assay was carried out immediately after the addition of urea.

[b] Incubation for the appropriate time in the absence of urea.

[c] Figures derived by interpolation.

DISCUSSION

The enzymic capacities of the two forms of tyrosinase which have been studied appear not to differ significantly, insofar as their substrate specificities, K_S values, response to inhibitors, and pH optima are

TABLE VIII

EFFECT OF FORMAMIDE UPON THE ACTIVITY OF THE STABLE (T^S) AND LABILE (T^L) FORMS OF TYROSINASE FROM *NEUROSPORA*

The substrate used was 7.1×10^{-5} *M* L-Dopa.

Formamide concentration	Minutes in formamide	Δ opt. dens. × 10^3		Per cent control velocity	
		T^S	T^L	T^S	T^L
M					
0	—	143	103	100	100
3.6×10^{-1}	0[a]	114	79	80	77
0	5[b]	130	86	100	100
3.6×10^{-1}	5	70	48	54	56
1.0	0[a]	77	60	54	59
1.0	5	69	37	53	44
1.8	0[a]	61	46	43	45
1.8	5	53	25	41	29
3.2	0[a]	40	28	28	27
3.2	5	31	10	24	12

[a] The assay was carried out immediately after the addition of formamide.

[b] Incubated for 5 min. in absence of formamide.

concerned. These observations extend the work begun by Horowitz and Fling (9) and confirm their conclusion that "functionally, the two enzymes are indistinguishable" (7).

That there may be differences which affect secondary and tertiary structure is borne out by the experiments in which the unfolding agents urea and formamide were used. These substances have a consistently greater inhibitory effect on the thermolabile form of the enzyme (T^L) than upon the stable one (T^S). This difference, along with those in thermal tolerance and electrophoretic mobility, points in the direction of a structural modification which alters the number and/or kind of bonds to which the folding characteristic of proteins is attributable.

The substrate specificity of *Neurospora* tyrosinase is roughly parallel to that of polyphenolases described from other organisms. However, there are differences in the relative effectiveness of the substrates. Thus, although Dopa is a better substrate for mushroom and *Neurospora* tyrosinase than is chlorogenic acid, the latter is the best substrate for the sweet potato enzyme (4). Catechol is oxidized about as rapidly as Dopa by the *Neurospora* enzyme, but reaction–inactivation is extremely rapid, making accurate determinations of rate very difficult. This explains why manometric techniques (15) failed to reveal the activity of *Neurospora* tyrosinase on catechol because measurements must be made within the first minute after the reaction is initiated and low concentrations of substrate must be used. Phenyl-4-catechol is the best substrate of those tried and appears to be used by other fungal tyrosinases as well (18).

Although the conclusions based upon the comparison of the activity of the two forms of tyrosinase are not affected by the reliability of the measurements with the ascorbate technique, the absolute rates and the K_S values are. The tests outlined in the section on *Methods* revealed that the method is reliable for use with diphenols, in agreement with other workers who have found that ascorbate has no effect upon the oxidation of these substrates by tyrosinase (11, 17, 19). However, the significance of the rates of oxidation of monophenols is not at all certain. Thus, it has been found that ascorbate increases the rate of oxidation and disappearance of tyrosine, especially in the early stages of the reaction (12–14). Moreover, part of the oxidation of ascorbate is coupled to the shuttle involving catechol and *o*-quinone, and the fraction of the oxidation due to monophenolase activity remains undetermined. In addition, changes in substrate concentration, as well as in the kind of substrate, may affect the proportion of the oxidation due to monophenolase activity. Therefore, the K_S values for the enzyme with tyrosine (Table II) are to be questioned, as are the absolute rates for the oxidation of monophenols listed in Table III. However, it is worth noting that no technique yet evolved provides unequivocal values for the oxidation of monophenols. The meaning of such rates is obscured by the oxidation of diphenols which is a concomitant of the initiation of the reaction. In fact, it has been suggested (14) that "In the absence of reducing agents, maximum activation is not obtained and . . . true phenolase activities can only be found in the

presence of reducing agents." In any event, the conclusion that the enzymes from T^S and T^L do not differ in their response to the substrates tried appears to be warranted, whether or not the absolute rates for the oxidation of monophenols are reliable.

As was mentioned in the section on *Methods*, the lag period in the oxidation of tyrosine is present when either the crude or purified enzyme is used in the ascorbate system. On the other hand, it has been shown[3] that when the oxidation of this substrate by purified enzyme systems is studied directly by the accumulation of halochrome, the lag period is eliminated. Furthermore, these results differ from those of others (3, 13, 14) in that the lag period has been reported to be removed by reducing agents like ascorbate. The resolution of these contradictions must await clarification of the nature of the lag period itself.

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