Mammalian Tyrosinase

A COMPARISON OF TYROSINE HYDROXYLATION AND MELANIN FORMATION

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1. Melanosomal tyrosinase was isolated from normal C57B1 mice, and a comparison of the tyrosine-hydroxylation and dopa (3,4-dihydroxyphenylalanine)-oxidation activities of this enzyme was made. 2. The results indicate that in the absence of dopa cofactor, this enzyme is capable of tyrosine hydroxylation, but with very little subsequent dopa oxidation and melanin formation. 3. This mechanism of enzyme action may play an important role in the intracellular regulation of melanin formation. 4. Further, dopa appears to act as a positive allosteric effector for tyrosine hydroxylation by tyrosinase, in addition to its known activity as a hydrogen donor for the reaction.

Tyrosinase (EC 1.14.18.1), the enzyme responsible for melanin synthesis, has been described in a variety of tissues in both the plant and animal kingdoms. Tyrosinase is a bifunctional enzyme in that it catalyses both the hydroxylation of tyrosine to dopa (L-3,4-dihydroxyphenylalanine) and the oxidation of dopa to dopaquinone (L-3,4-dioxyphenylalanine) (Scheme 1). In addition, it is known that tyrosine hydroxylation by tyrosinase is dependent on the presence of dopa in catalytic quantities. Initial methods for assay of the enzyme used manometry and/or spectroscopy, among other techniques (Lerner *et al.*, 1949; Lerner, 1955). However, none of these earlier tyrosinase assays was adequate to separate and characterize these different enzymic functions. Further, the specificity of identification of the products measured with those techniques in crude enzyme preparations was inadequate.

More recently, sensitive and specific radiochemical assays have been developed, which are of two types: (a), those that utilize [³H]tyrosine and measure the formation of ³H₂O as a by-product of tyrosine hydroxylation to dopa (Nagatsu *et al.*, 1964; Pomerantz, 1964; Oikawa *et al.*, 1972); (b), those that use [¹⁴C]tyrosine and measure the formation of acid-insoluble radioactive melanin and melanin precursors (Kim & Tchen, 1962; Chen & Chavin, 1965). Insofar as the measurement of tyrosinase activity is concerned, both assays assume that all tyrosine hydroxylated is used in dopa oxidation and subsequent melanin production. Our results suggest that this condition is not obligatory, and that with different dopa cofactor concentrations, the amount of tyrosine hydroxylated differs from that converted into melanin. This study on mouse tyrosinase from normal melanocytes compares these radiological methods of assay, and deter-

mines more specifically the role of dopa in tyrosinase activation and melanin formation. In addition, both these assays were adapted to micro-assay volumes and standardized with regard to substrate concentrations.

Materials and Methods

Materials

All solutions were made in distilled, de-ionized water; all reagents were of the highest purity available. L-Dopa, D-dopa, DL-dopa, dihydroxyfumaric acid, ascorbic acid, dopSer (DL-*threo*-3,4-dihydroxyphenylserine), 3,4-dihydroxyphenylacetic acid, dopamine (3-hydroxytyramine), bovine serum albumin, chloramphenicol, cycloheximide, penicillin G and diethyldithiocarbamate were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); Celite 545, Norit A and alumina gel were from Fisher Scientific Co. (Fair Lawn, NJ, U.S.A.); sodium monobasic and dibasic phosphates and trichloroacetic acid were from J. T. Baker Chemical Co. (Phillipsburg, NJ, U.S.A.); ultrapure sucrose was from Schwarz/Mann (Orangeburg, NY, U.S.A.); Bio-Beads SM-2 were from Bio-Rad Laboratories (Richmond, CA, U.S.A.); Triton X-100 was from Research Products International (Elk Grove Village, IL, U.S.A.); Aquasol and 2,5-diphenyloxazole were from New England Nuclear (Boston, MA, U.S.A.). Radioactive substrates (L-[U-¹⁴C]tyrosine, L-[3,5-³H]tyrosine, and L-[U-¹⁴C]-dopa) were obtained from both Amersham/Searle (Arlington Heights, IL, U.S.A.) and Schwarz/Mann; the tyrosine compounds were purified from possible dopa contamination by passage through a column (1 cm × 20 cm) of alumina gel in 1.0 M-ammonium acetate buffer, pH 6.1 (Pomerantz, 1966).

Scheme 1. Metabolic pathway of tyrosine conversion into melanin

The classic scheme of Raper (1928) and Mason (1948). Although deviations from this pathway are known to occur in melanin formation [for a review, see Swan (1974)], this metabolic path adequately represents the majority of product formations.

Enzyme preparation

The fraction containing melanin granules was isolated from normal black (C57B1) mouse skin by differential and density-gradient centrifugation as described previously (Hearing & Lutzner, 1973; Klingler *et al.*, 1976). All steps were carried out at 4°C. Briefly, tissues were homogenized in 0.1M-

sodium phosphate buffer, pH7.4, the sample was centrifuged for 5min at 200g, and the supernatant was further centrifuged at 10000g for 10min; the resulting pellet was resuspended in the phosphate buffer. This suspension was again centrifuged at 200g for 5min, after which the supernatant was centrifuged at 10000g for 10min. The resuspended pellet was

layered on 30% (w/v) sucrose and centrifuged at 15000g for 10min; this pellet was resuspended in phosphate buffer and washed through 30% sucrose again; the resulting melanin-granule pellet was shown by electron microscopy to be free of contaminating cellular organelles. Melanosomal tyrosinase was solubilized from this purified fraction with 1% (v/v) Triton X-100 for 1 h at 4°C. Excess of Triton X-100 was then removed by the procedure of Holloway (1973), the suspension was centrifuged at 10000g for 10min, and the supernatant passed through a Millipore HA filter (0.45 μ m pore size). Enzyme samples were always prepared fresh and were not frozen.

Tyrosinase activity determinations

[14 C]Tyrosine assay. Modifications of the techniques originally described by Kim & Tchen (1962) and Achazi & Yamada (1972) were used. Each assay (final volume 25 μ l) contained in 0.1M-phosphate buffer, pH 7.4, 10 μ l of enzyme preparation, 5 μ l of [14 C]tyrosine (25 μ Ci/ml, sp. radioactivity 100 mCi/mmol), 5 μ l of a solution containing chloramphenicol (1 mg/ml), cycloheximide (1 mg/ml), penicillin G (1000 units/ml), bovine serum albumin (0.1 mg/ml), and 5 μ l of various other agents as specified in the text. The assays were run as a routine in duplicate at 37°C for 1 h, or for times specified in the legend of Fig. 1 and those of the Tables. After incubation, the samples were placed on ice, and 20 μ l samples removed to Whatman 3MM filter-paper discs. The filters were washed as follows: one 15 min wash with 3 litres of 5% (w/v) trichloroacetic acid (containing unlabelled 50 mM-tyrosine), two 15 min washes with 3 litres of 5% trichloroacetic acid, two 5 min washes each in 1 litre of aq. 99% (v/v) ethanol, one 5 min wash in 1 litre of acetone. The filters were then allowed to dry in air, and were counted for radioactivity in 0.4% (w/v) 2,5-diphenyloxazole in toluene (with 70% efficiency). As a routine the final concentration of tyrosine was 0.05 mM, and under these conditions the reaction is dependent on enzyme and substrate concentration. Controls ordinarily used included blanks from which the substrate and/or enzyme were omitted, boiled enzyme preparations, and diethyldithiocarbamate-inhibited controls.

[14 C]Dopa assay. Incubations were set up as described for the [14 C]tyrosine assay, except for the label, which was [14 C]dopa (7.5 μ Ci/ml, sp. radioactivity 30 mCi/mmol). After the incubation time, samples were placed on ice, and 20 μ l samples placed on Whatman 3MM filter pads, which were then processed as for the [14 C]tyrosine assay.

[3 H]Tyrosine assay. Incubations were set up as described for the [14 C]tyrosine assay, except for the label, which was [3 H]tyrosine (50 μ Ci/ml, sp. radioactivity 200 mCi/mmol). After incubation, the samples were placed on ice, and 20 μ l samples were removed

into 0.98 ml of 5% (w/v) trichloroacetic acid containing approx. 100 mg of Celite 545 and 100 mg of Norit A. These were shaken at room temperature (27°C) for a minimum of 90 min, centrifuged at 1000g for 10 min, and 200 μ l portions removed and counted for radioactivity in Aquasol (at 30% efficiency). Appropriate corrections were made to facilitate direct comparisons with the [14 C]tyrosine assays.

The validity of both these assays for measuring the specific reaction that they purport to measure has been well documented (Kim & Tchen, 1962; Nagatsu *et al.*, 1964; Pomerantz, 1964; Chen & Chavin, 1965; Oikawa *et al.*, 1972), and will not be discussed here. Results are reported as pmol of substrate utilized in the time-period assayed. In the [3 H]tyrosine assay, this provides a measure of the amount of tyrosine hydroxylated (formation of 3 H₂O from the 3- 3 H). In the 14 C assays, the amount of tyrosine or dopa converted into acid-insoluble tyrosine derivatives, which consist of the indolequinones and melanin (Oikawa *et al.*, 1972), is measured. A unit of tyrosinase is defined as the amount of enzyme that metabolizes 1 μ mol of tyrosine/min, measured by the [14 C]tyrosine assay, in the presence of 0.005 mM-dopa. Experiments were performed a minimum of three times in duplicate and the results of a representative experiment are presented; backgrounds were subtracted from all values reported.

Results and Discussion

[3 H]Tyrosine assay

There are several obvious disadvantages of the [3 H]tyrosine assay. First, the rate of non-enzymic 3 H exchange with water is significant, and thus the background may be a problem (in these experiments, the background was usually about 10% of the observed activity). Secondly, a considerable amount of the 3 H label can be located in positions other than C-3 and C-5 of the tyrosine and thus is unavailable for measurement. Perhaps most importantly, although the stoichiometry of the release of the 3- 3 H during tyrosine hydroxylation by melanoma tyrosinase has been shown, some of the 5- 3 H is released on polymerization to melanin (Pomerantz, 1966; Oikawa *et al.*, 1972). Ascorbate can be added to circumvent the latter problem, as it immediately reduces dopa-quinone back to dopa, thus preventing melanin formation and subsequent release of the 5- 3 H; on the other hand, ascorbate increases the rate of tyrosine hydroxylation slightly and thus affects enzyme activity. Notwithstanding these considerations, the advantages of the [3 H]tyrosine assay include its sensitivity, and its direct measurement of tyrosine-hydroxylating activity, rather than dependence on the formation of products.

In Fig. 1(a), the incorporation characteristics of this assay over a 48 h period in the presence of various

Fig. 1. Time-course of melanin formation by tyrosinase as determined by: (a) L-[3,5-³H]tyrosine assay; (b) L-[U-¹⁴C]tyrosine assay

Assays were carried out as described in the Materials and Methods section, with various concentrations of dopa cofactor. Assays were incubated at zero time, and at the times indicated, samples were removed and processed as described in the text. Dopa cofactor concentrations: $\diamond$, 0.05 mM; $\circ$, 0.01 mM; $\square$, 0.005 mM; $\triangle$, 0.001 mM; $\blacklozenge$, 0.0005 mM; $\bullet$, 0.0001 mM; $\blacksquare$, 0.0 mM.

amounts of dopa are reported. Rates of tyrosine hydroxylation were linear with time initially, and slowed after 50% of the available substrate had been utilized. It is noteworthy that mammalian tyrosinase is extremely stable, and that after 24 h at 37°C, more than 85% of the activity remains.

[¹⁴C]Tyrosine assay

The disadvantages of the [¹⁴C]tyrosine assay include, first, the reliance on the formation of acid-insoluble products rather than the immediate products, dopa and/or dopaquinone (Scheme 1); the

[¹⁴C]tyrosine assay thus relies on the conversion of the newly formed [¹⁴C]dopa into [¹⁴C]dopaquinone rather than the utilization by the enzyme of exogenously added dopa. Secondly, the loss of one ¹⁴C atom occurs during the decarboxylation step of dopachrome to 5,6-dihydroxyindole. The principal advantages of this assay include ease of execution, good counting efficiency and an extremely low rate of non-enzymic conversion of tyrosine into dopa (in these experiments, the background was usually less than 2% of the observed radioactivity).

With identical amounts of enzyme and substrates

as reported in Fig. 1(a) for the [³H]tyrosine assay, the data on tyrosinase activity as measured by the [¹⁴C]tyrosine method are presented in Fig. 1(b). Little or no incorporation of [¹⁴C]tyrosine into [¹⁴C]-melanin was seen in the absence of dopa, even after 48 h. An initial lag period was observed at dopa concentrations of 0.005, 0.001 and 0.0005 mM. At higher dopa concentrations (0.01 and 0.05 mM), and until more than 50% of the label had been used incorporations were nearly linear with time.

Comparison of methodology

Although each of these assays is superior to earlier assay methodology with regard to sensitivity and specificity of measurement, it has been demonstrated that neither alone truly defines actual tyrosinase activity. The adjustment of both the [¹⁴C]tyrosine and the [³H]tyrosine assays to identical concentrations of substrate, dopa cofactor and other added compounds, and at the same time, decreasing the volume of these assays to 25 μ l, has many obvious advantages. Perhaps the most important of these is the direct comparison then available between the assays. In addition, one of the major objections to the [¹⁴C]tyrosine assay has been the long time-period of incubation (18 h) required (Chen & Chavin, 1969). We have increased the specific radioactivity of the labelled substrates, and thus decreased the time necessary for incubation, obviating this problem (incubation times of 15–30 min are adequate). A pH of 7.4 and temperature of 37°C are optimum for tyrosinase activity (Chen & Chavin, 1965; Chen, 1975) and are used in these assays. Further, the substrate (tyrosine) concentration has been placed at 0.05 mM. Concentrations above this have been found to be inhibitory, and this was the highest permissible tyrosine concentration to use without significant substrate inhibition.

Our results indicated that even 0.05 mM-tyrosine was capable of a slight amount of enzyme inhibition, which resulted in the lag period characteristic for tyrosinase (Fig. 1b). The routine tyrosine concentration used in both the [³H]tyrosine and [¹⁴C]tyrosine assays as originally described was 0.5 mM or above, which explains the lag period found by previous investigators (Lerner *et al.*, 1949; Pomerantz, 1964). Evidence against the apparent substrate inhibition resulting from a tyrosine contaminant includes the kinetic determination that the inhibition is competitive with dopa, and reversible, and the fact that assays of the labelled tyrosine by several different solvent systems on paper chromatography indicate greater than 99% purity of the tyrosine.

Studies with cofactor analogues

The effects of various cofactor analogues on tyrosinase activity as measured by both assays are reported in Table 1. None of the agents tested at any concentration increased enzyme activity to the same extent as did lower concentrations of DL-dopa. Also, in the presence of dopa, both dihydroxyfumaric acid and 3,4-dihydroxyphenylacetic acid increased tyrosinase activity slightly as determined by the [³H]-tyrosine assay, but the observed activity as measured by the [¹⁴C]tyrosine assay was unaffected or decreased in each case. As mentioned above, the effect of added hydrogen donors is to reduce dopaquinone back to dopa, thereby preventing melanin formation; this in turn raises the dopa concentration slightly and thus the rate of tyrosine hydroxylation, while lowering the observed rate of melanin formation. This results in the increase in tyrosine hydroxylation as noted by the [³H]tyrosine assay, and the decrease in melanin formation as determined by the [¹⁴C]tyrosine assay.

These results highlighted a complication of the

Table 1. Effects of cofactor analogues on demonstrable tyrosinase activity by using [3,5-³H]- and [U-¹⁴C]-tyrosine assays

Assay conditions were carried out as described in the Materials and Methods section, with various concentrations of cofactors. The results are reported as pmol of tyrosine metabolized by tyrosinase (4×10^{-7} units) in 1 h. Percentage standard deviations were all 10% or less; *n* = 4. A standard curve was drawn by using DL-dopa as cofactor, and the relative efficiency of the substrate analogues was then determined by dividing the concentration of substrate used by the concentration of DL-dopa which gave an equivalent stimulation of tyrosinase activity.

Cofactor (0.05 mM)	Assay	Dopa (mM)	...	Tyrosinase activity				Relative efficiency
				0	0.0005	0.005	0.05	
Buffer	[³ H]Tyr			9	20	96	237	5
	[¹⁴ C]Tyr			1	6	24	57	0
Dihydroxyfumarate	[³ H]Tyr			4	20	103	244	0
	[¹⁴ C]Tyr			0	3	24	56	0
Dihydroxyphenylacetic acid	[³ H]Tyr			7	27	93	254	0
	[¹⁴ C]Tyr			1	4	22	57	0
DopSer	[³ H]Tyr			96	121	159	240	10
	[¹⁴ C]Tyr			17	19	25	36	5
Dopamine	[³ H]Tyr			125	151	164	246	18
	[¹⁴ C]Tyr			33	35	41	54	20

[^{14}C]tyrosine assay. In the presence of reducing agents, the dopaquinone produced by tyrosinase was reduced back to dopa, thereby lowering the accumulation of labelled acid-insoluble products. Some authors have interpreted results of this kind to indi-

cate that these agents are inhibitors of tyrosinase, rather than of melanin formation (Chen & Chavin, 1972). This problem was not evident with the [^3H]tyrosine assay; therefore in the presence of active hydrogen donors (particularly in crude preparations),

Table 2. *Ability of L-dopa and D-dopa to act as tyrosinase cofactors*

Radiochemical assays were carried out as described in the Materials and Methods section, with various amounts of L-dopa, D-dopa or DL-dopa as cofactors. The results are reported as pmol of tyrosine metabolized in 1 h (mean $\pm$ s.d., $n = 4$). A standard curve was drawn by using L-dopa as cofactor, and the relative efficiency of D-dopa and DL-dopa was then determined by dividing the concentration of cofactor used by the concentration of L-dopa that gives an equivalent stimulation of tyrosinase activity (1.14×10^{-6} units).

Cofactor (mM)	[^{14}C]Tyrosine		[^3H]Tyrosine	
	(pmol)	Relative efficiency	(pmol)	Relative efficiency
L-Dopa (0.05)	110.8 $\pm$ 18	100	297.4 $\pm$ 12	100
(0.01)	91.5 $\pm$ 7	100	241.5 $\pm$ 17	100
(0.005)	68.6 $\pm$ 8	100	201.6 $\pm$ 40	100
(0.001)	20.0 $\pm$ 3	100	60.1 $\pm$ 14	100
(0.0005)	8.9 $\pm$ 0.6	100	17.0 $\pm$ 4	100
(0.0001)	1.1 $\pm$ 0.1	100	6.4 $\pm$ 2	100
D-Dopa (0.05)	48.5 $\pm$ 7	6	229.4 $\pm$ 12	16
(0.01)	23.0 $\pm$ 2	13	80.6 $\pm$ 9	13
(0.005)	12.6 $\pm$ 1	13	46.0 $\pm$ 4	13
(0.001)	2.5 $\pm$ 0.3	17	2.6 $\pm$ 0.5	11
DL-Dopa (0.05)	82.8 $\pm$ 9	18	314.4 $\pm$ 25	61
(0.01)	63.4 $\pm$ 7	51	196.8 $\pm$ 43	63
(0.005)	44.8 $\pm$ 4	60	129.7 $\pm$ 23	59
(0.001)	9.9 $\pm$ 1	53	21.7 $\pm$ 2	30

Table 3. *Kinetic parameters of mouse tyrosinase*

Radiochemical assays were carried out as described in the Materials and Methods section, at various concentrations of dopa cofactor. Substrate concentrations used for the estimation of kinetic values ranged from 0.005 mM- to 0.0001 mM-tyrosine, and a minimum of 20 data points were used to construct each curve. Kinetic values reported were estimated by least-squares weighted linear regression analysis of Eadie (1942)–Hofstee (1959) plots; 2.73×10^{-6} units of tyrosinase were used; values reported are $\pm$ s.d., $n = 3$.

L-[3,5- ^3H]Tyrosine	$10^7 \times V$ (mol/h)	$10^5 \times K_m$ (M)
0.05 mM-Dopa	1.100 $\pm$ 0.80	3.300 $\pm$ 0.69
0.005 mM-Dopa	2.200 $\pm$ 0.60	1.700 $\pm$ 0.60
0.001 mM-Dopa	1.100 $\pm$ 0.90	1.000 $\pm$ 0.50
0.0005 mM-Dopa	1.100 $\pm$ 0.30	1.200 $\pm$ 0.40
0.0001 mM-Dopa	0.350 $\pm$ 0.13	0.320 $\pm$ 0.25
0.00005 mM-Dopa	0.460 $\pm$ 0.16	0.520 $\pm$ 0.31
0.0 mM-Dopa	0.760 $\pm$ 0.17	1.200 $\pm$ 0.40
L-[U- ^{14}C]Tyrosine		
0.05 mM-Dopa	0.770 $\pm$ 0.02	0.140 $\pm$ 0.02
0.005 mM-Dopa	0.680 $\pm$ 0.09	0.130 $\pm$ 0.02
0.001 mM-Dopa	0.190 $\pm$ 0.03	0.067 $\pm$ 0.01
0.0005 mM-Dopa	0.110 $\pm$ 0.01	0.066 $\pm$ 0.01
0.0001 mM-Dopa	0.041 $\pm$ 0.01	0.064 $\pm$ 0.02
0.00005 mM-Dopa	0.032 $\pm$ 0.01	0.047 $\pm$ 0.01
0.0 mM-Dopa	0.028 $\pm$ 0.01	0.190 $\pm$ 0.05
L-[U- ^{14}C]Dopa		
0.0 mM-Tyrosine	58.00 $\pm$ 6	9.5 $\pm$ 2

the [^{14}C]tyrosine assay may yield misleading results. It is also clear from the results reported in Table 1 that the other cofactor analogues studied (dopSer and dopamine) were not effective in activating tyrosinase, since even high concentrations (0.05 mM) did not result in large activations in activity as did 0.005 or 0.05 mM-DL-dopa.

The ability of the D isomer of dopa to act as cofactor in place of L-dopa was also examined (Table 2). At each concentration, D-dopa was only approx. 15% as effective as L-dopa as cofactor, and these data are corroborated by the cofactor activity exhibited by DL-dopa.

Tyrosinase activity and catalytic sites

The kinetics of tyrosine hydroxylation and melanin formation in the presence of various concentrations of dopa are presented in Table 3. The kinetic measurements of V (reaction velocity) and K_m as determined by the two radiochemical assays varied by as much as an order of magnitude. Increasing the dopa concentration leads to only a twofold increase in the V of tyrosine hydroxylation, but increased the V of melanin formation by about 30-fold. Further, at low dopa concentrations, a decrease in the K_m for tyrosine by both assays is noted. Thus dopa at low concentrations acts as a non-competitive activator of tyrosine hydroxylation and melanin formation.

Since L-dopa is also a substrate for tyrosinase, it was decided to examine the relation of the catalytic site(s) for both tyrosine and dopa as substrates. Previous workers have presented kinetic evidence that there are probably at least two distinct sites involved in the binding of tyrosine and dopa to tyrosinase, although it was not known whether the dopa-

binding site functioned as an enzymic site for dopa oxidation (Shimao, 1962; Pomerantz & Warner, 1967). The present studies, involving the use of labelled and unlabelled substrates in a competitive manner, indicate (Table 4) that there are two different catalytic sites for tyrosine and dopa. In fact, at the catalytic concentrations studied (0.005 mM and below) there is little, if any, competition for substrates between them. These data support the view that the enzyme has two distinct catalytic sites, one for tyrosine hydroxylation and another for dopa oxidation. It further shows that at the lower dopa concentrations there is little leakage of the dopa formed *de novo* by tyrosinase from tyrosine to the media before it is shifted to the dopa catalytic site and oxidized to dopaquinone. Additional evidence for the two catalytic sites can be derived from the fact that although dopa is a close substrate analogue to tyrosine, its K_m (1×10^{-3} M) as a substrate is two orders of magnitude higher than that of tyrosine (cf. Table 3). As D-dopa was only 15% as effective as L-dopa as cofactor (Table 2), tyrosinase is very specific for L-dopa as cofactor for tyrosine hydroxylation.

Tyrosinase activation by dopa cofactor

Table 5 shows the amount of tyrosine that was hydroxylated and that which was ultimately converted into melanin. At a dopa concentration of 0.005 mM, the amount of tyrosine hydroxylated that was actually converted into melanin increased with time; namely from 21% after 1 h to 45% after 5 h, and 62% after 8 h. At a tyrosine/dopa molar ratio of 1:1 (0.05 mM-dopa), roughly the same values were found. Since the 1250 pmol of [^{14}C]melanin reported represented total utilization of the tyrosine in the

Table 4. *Tyrosine versus dopa as tyrosinase substrates*

Radiochemical assays were carried out under the conditions described in the Materials and Methods section, by using various concentrations of [^{14}C]dopa or unlabelled dopa as cofactor. The results are reported as pmol of labelled substrates (mean $\pm$ s.d., n in parentheses) converted into acid-insoluble melanin precursors/h. '%' is the ratio (dopa converted into melanin/tyrosine converted into melanin) at any particular concentration; 2.57×10^{-6} units of tyrosinase were used.

Substrate (0.05 mM)	Cofactor (mM)	Conversion	
		(pmol)	(%)
1 [^{14}C]Tyrosine	Dopa (0.05)	181.4 $\pm$ 22 (8)	—
2	Dopa (0.01)	164.7 $\pm$ 13 (8)	—
3	Dopa (0.005)	153.9 $\pm$ 16 (12)	—
4	Dopa (0.001)	60.8 $\pm$ 7 (12)	—
5	Dopa (0.0005)	35.4 $\pm$ 5 (12)	—
6	Dopa (0.0001)	9.1 $\pm$ 1 (12)	—
7 Tyrosine	[^{14}C]Dopa (0.05)	220.8 $\pm$ 15 (8)	122
8	[^{14}C]Dopa (0.01)	48.1 $\pm$ 7 (8)	29
9	[^{14}C]Dopa (0.005)	24.7 $\pm$ 2 (8)	16
10	[^{14}C]Dopa (0.001)	4.5 $\pm$ 0.5 (8)	7
11	[^{14}C]Dopa (0.0005)	2.0 $\pm$ 0.3 (8)	6
12	[^{14}C]Dopa (0.0001)	0.2 $\pm$ 0.0 (8)	2

Table 5. *Melanin formation as a function of time*

Assays were carried out as described in the Materials and Methods section, by using various concentrations of dopa cofactor. The results are reported as pmol of substrate metabolized ($\pm$ S.D., $n = 4$) by tyrosinase (7.83×10^{-7} units). '%' indicates the amount of tyrosine hydroxylated that was converted into melanin in the noted time.

Incubation time ...	Tyrosine metabolized			
	1 h	3 h	5 h	8 h
Without dopa				
[14 C]Tyr	0 $\pm$ 2	2 $\pm$ 1	2 $\pm$ 1	4 $\pm$ 1
[3 H]Tyr	26 $\pm$ 6	48 $\pm$ 3	68 $\pm$ 6	97 $\pm$ 7
Melanin (%)	0	4	3	4
With 0.0005 mM-dopa				
[14 C]Tyr	7 $\pm$ 1	16 $\pm$ 2	47 $\pm$ 2	148 $\pm$ 6
[3 H]Tyr	41 $\pm$ 4	99 $\pm$ 17	265 $\pm$ 17	858 $\pm$ 64
Melanin (%)	17	16	18	17
With 0.005 mM-dopa				
[14 C]Tyr	47 $\pm$ 5	368 $\pm$ 32	747 $\pm$ 93	1002 $\pm$ 88
[3 H]Tyr	222 $\pm$ 31	1174 $\pm$ 73	1662 $\pm$ 208	1624 $\pm$ 26
Melanin (%)	21	31	45	62
With 0.05 mM-dopa				
[14 C]Tyr	153 $\pm$ 15	626 $\pm$ 39	922 $\pm$ 35	1074 $\pm$ 40
[3 H]Tyr	706 $\pm$ 35	1604 $\pm$ 140	1777 $\pm$ 267	1628 $\pm$ 265
Melanin (%)	22	39	52	66

assay, the value of 60% melanin formation reflects the maximum loss of 5- 3 H due to polymerization, as reported by Hempel (1966) and Oikawa *et al.* (1972). The utilization of more than 100% of the [3 H]tyrosine (1250 pmol) is also evident in Fig. 1(a). These results showed that all tyrosine hydroxylated at these dopa concentrations was ultimately converted into melanin. At a lower tyrosine/dopa molar ratio of 1:100 (0.0005 mM-dopa), a different picture was presented. In the time-range 1–8 h, the amount of tyrosine hydroxylated that was converted into melanin was maintained at about 17%. These results indicate that, although tyrosine may be hydroxylated to dopa, the formation of melanin and acid-insoluble melanin precursors did not necessarily follow. This was also seen in the experiments in which dopa was omitted. Little incorporation into [14 C]melanin was found, although small amounts of tyrosine hydroxylation did occur, and even after 8 h, only 4% of the tyrosine hydroxylated had been converted into melanin.

This relationship indicates that the percentage of melanin formed after tyrosine hydroxylation was dependent on dopa concentration. This in turn could have been due to one or both of the following causes: (a) the effect of dopa on the tyrosinase enzyme molecule, which resulted in increased melanin formation (this must be distinguished from the hydrogen-donor role of dopa in hydroxylating tyrosine), or (b) the direct participation of exogenous dopa in melanin formation and the subsequent binding of [14 C]tyrosine to this. Evidence against the latter came from several experiments in which dopa at various concen-

trations was allowed to autoxidize at pH 8.0 at 37°C in the presence of [14 C]tyrosine. At various time-intervals, samples were removed and placed on Whatman 3MM filter discs, then processed for [14 C]-melanin as detailed in the Materials and Methods section. There was no significant binding of the [14 C]-tyrosine to the synthetic melanin, even after 5 h, when most of the dopa had been autoxidized.

One possible explanation for the observed effect of dopa on increased melanin formation is a conformational change of tyrosinase elicited by dopa, which might align the catalytic sites for tyrosine and dopa, leading to increased efficiency in the coupling of tyrosine hydroxylation with dopa oxidation. Such a mechanism would also allow the increase in dopa concentration that was necessary for significant melanin formation. The major proportion of dopa synthesized from tyrosine would not be converted into melanin until the dopa concentration was raised enough to optimize enzymic activity. This is a teleologically simple, but effective, control mechanism. This apparent mechanism of enzyme action may explain, at least in part, the lack of melanin formation observed in the Golgi apparatus and smooth endoplasmic reticulum in melanocytes, even though active tyrosinase can be detected in these organelles (Seiji *et al.*, 1963; Maul & Brumbaugh, 1971; Eppig & Dumont, 1972; Hearing, 1973). The initial lack of an adequate dopa concentration would minimize any actual formation of melanin while the enzyme was present in these organelles in transit to the melanosome. Only when the enzyme was *in situ* would ade-

quate dopa concentrations be attained to result in measurable amounts of melanin elaboration.

In the present study it was found that dopa (at low concentrations) was not a competitive inhibitor of tyrosine hydroxylation (Table 3), but rather was a non-competitive activator for tyrosine binding and reaction velocity. At about 0.0001 mM-dopa, the effect on the K_m was optimized. At 0.05 mM-dopa, the V was higher, but the K_m was also increased, probably owing to competitive inhibition. This activation by dopa resulted in a 30-fold stimulation of melanin formation. Our composite data are most easily explained by tyrosinase having at least one site for tyrosine binding and enzymic function, and at least one other where dopa can effect its change on the affinity of the enzyme for tyrosine. This latter site may or may not be enzymically functional in dopa oxidation, although some evidence suggested that it did function in dopa oxidation. As mentioned above, dopa is a good hydrogen donor, and its acceleration of reaction velocity could possibly be ascribed in part to that, but its effect on the affinity of the enzyme for tyrosine is not so readily explained. When several other similar hydrogen donors (D-dopa, dopSer, 3,4-dihydroxyphenylacetic acid and dopamine) were checked, no such activation resulted. Thus it seems very likely that dopa induces a conformational change in normal tyrosinase which not only makes the binding of tyrosine more favourable to the enzyme, but also greatly increases the velocity.

Such a conformational activation of the enzyme would also explain the inhibition of activity caused at higher concentrations of tyrosine. Since the normal binding site for dopa might be successfully competed for by tyrosine at high concentrations, the enzyme would then assume its more inactive conformation and the rate of melanin synthesis would diminish. Higher concentrations of dopa would again reverse this action; such a reversal was reported by Pomerantz (1966).

Thus we conclude that the amount of hydroxylated tyrosine that is converted into melanin is not constant, but varies slightly as a function of time of incubation and greatly as a function of dopa concentration. As a corollary to this, the two functions of tyrosinase (i.e. the hydroxylation of tyrosine to dopa and the oxidation of dopa to dopaquinone) are entirely distinct activities. In the presence of catalytic quantities of dopa there is good correlation between the two radiochemical assays, and since most assays are carried out in this manner, it may not make much difference

which assay is used. However, at the lower concentrations of dopa, extant intracellularly, inherent errors may occur when either one of these assays is used without complementation by the other.

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