[Chem. Pharm. Bull. 30(6)2094—2098(1982)]

Radioassay for Oxidation of Tyrosine by Tyrosinase using L-[Ring-^{3}H]tyrosine and L-[Carboxyl-^{14}C]tyrosine

Noriko Motohashi,* Harumi Eguchi, and Itsuhiko Mori

Kobe Women's College of Pharmacy, Motoyamakita-machi, Hihashinada-ku, Kobe, 658, Japan

(Received November 6, 1981)

Two intermediates involved in the synthesis of melanin from L-tyrosine by mushroom tyrosinase (EC 1.14.18.1) were assayed simultaneously by using L-[ring-^{3}H]tyrosine and L-[carboxyl-^{14}C]tyrosine. Tritium ions released from the 3-position of L-[3,5-^{3}H]tyrosine or the 6-position of L-[2,6-^{3}H]tyrosine were isolated as ^3H_2O and employed to determine the quantity of L-dopa or leucodopachrome, respectively (^3H_2O method). 5,6-Dihydroxyindole formed at the decarboxylation step was estimated from the radioactivity remaining after the evolution of $^{14}CO_2$ from L-[carboxyl-^{14}C]tyrosine ($^{14}CO_2$-release method). The lower limits of determination in the ^3H_2O and the $^{14}CO_2$-release methods were 0.1 pmol and nmol of the intermediates, respectively. The formations of L-dopa, leucodopachrome, and 0.1 5,6-dihydroxyindole were measured at various concentrations of L-tyrosine by both methods, and dopachrome was determined colorimetrically by measuring the absorbance changes at 475 nm. The maximum velocities were estimated from Lineweaver-Burk plots.

Keywords——radioassay tyrosine tyrosinase; tyrosine radioassay tyrosinase; tyrosinase tyrosine radioassay; L-[3,5-tritium]tyrosine; L-[2,6-tritium]tyrosine; L-[carboxyl-carbon 14]tyrosine

A sensitive assay is needed to clarify the reaction pathway to melanin from tyrosine catalyzed by tyrosinase. As shown in Chart 1, the reaction pathway has not yet been completely elucidated.[1] This has been due to the general complexity of the reaction, which has some enzymatically catalyzed steps and some which are not. The spectrophotometric assay of dopachrome, which is generally used, is not very sensitive because the molar absorptivity of dopachrome at 475 nm is 3700.[2] A radioassay with high sensitivity has been tested using L-[3,5-^{3}H]tyrosine[3] and L-3,4-[2,5,6-^{3}H]dihydroxyphenylalanine(dopa),[4] but the high radioac-

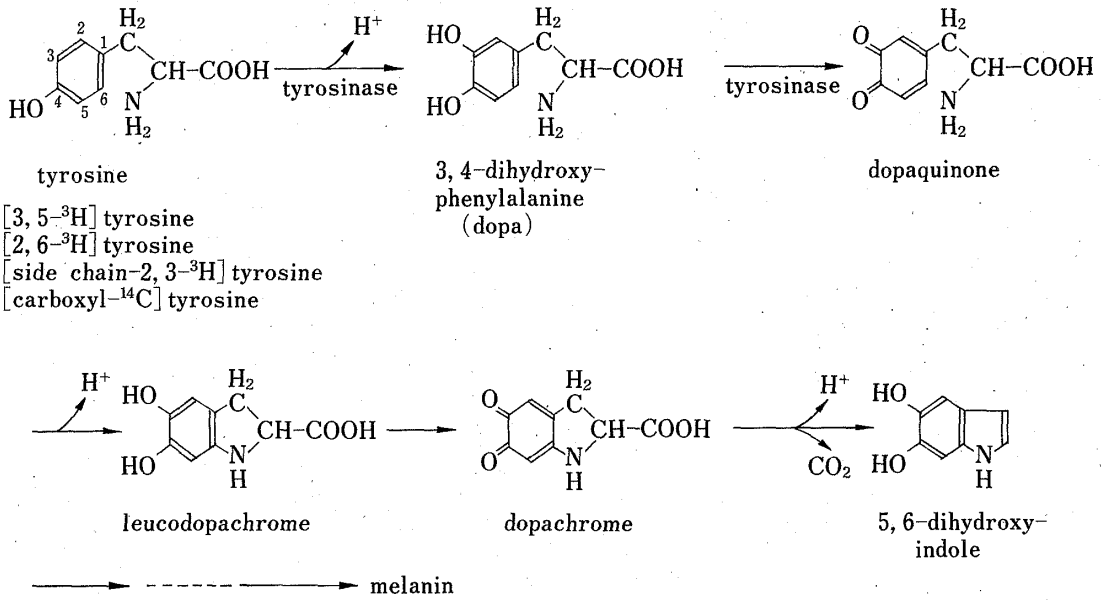

Chart 1. Scheme for the Production of Melanin by the Oxidation of Tyrosine[1]

tivity of the blank diminishes its sensitivity. This paper describes a simultaneous radioassay with a lower blank level of some intermediates formed from both [ring-^{3}H]tyrosine and [carboxyl-^{14}C]tyrosine by tyrosinase.

Experimental

Materials——Mushroom tyrosinase [EC 1.14.18.1, 2300 units/mg, grade III) was purchased from Sigma Chemical Company. Radioactive compounds, L-[3,5-^{3}H]tyrosine (40 mCi/μmol), L-[2,6-^{3}H]tyrosine (40 mCi/μmol), L-[side chain-^{3}H]tyrosine (10 mCi/μmol) and L-[carboxyl-^{14}C]tyrosine (57 mCi/mmol) as 2% ethanol solutions, sodium [^{14}C]carbonate and tritiated water, were obtained from Amersham. Each radioactive tyrosine solution was evaporated to dryness under a nitrogen atmosphere just before use, and the residue was dissolved in 25 mM potassium phosphate buffer, pH 6.8. The liquid scintillation counting medium was premixed scintillator, Dotite Scintisol EX-H (Dojindo Laboratories). All other reagents were of the highest purity commercially available.

Radiochemical Tyrosinase Assay by the $^{3}H_2O$ and $^{14}CO_2$-Release Methods——The reaction mixture consisted of 0.1 to 0.8 μmol of L-tyrosine containing either L-[3,5-^{3}H]tyrosine or L-[2,6-^{3}H]tyrosine (1 μCi) and L-[carboxyl-^{14}C]tyrosine (0.1 μCi), 100 units of mushroom tyrosinase and 25 μmol potassium phosphate buffer, pH 6.8, per milliliter. The reaction was initiated by the addition of tyrosinase and the mixture was incubated at 37°C. Blanks for each time point were incubated without tyrosinase. Two-milliliter portions were separated from the reaction mixture at appropriate times and divided into two aliquots after the addition of 0.4 ml of 40% metaphosphoric acid.

An aliquot was used to determine the amount of $^{3}H^{+}$ liberated through the formation of L-dopa or leucodopachrome (Chart 1). One point two milliliters of the reaction mixture was diluted to 5.0 ml with distilled water, and treated with 300 mg of charcoal (Norit A). After being left standing for 30 min, the solution was filtered and 1 ml of the filtrate was transferred to a 1.5 × 3.0 cm column of Dowex 50 W × 8 (H^{+} form, 50—100 mesh). The column was washed with distilled water to make a total of 10 ml. One milliliter of the eluate isolated as $^{3}H_2O$ was mixed with 15 ml of liquid scintillation medium, and the radioactivity was measured with a ^{3}H/^{3}HQ channel of a Packard TRI-CARB 3255 liquid scintillation counter. The initial radioactivity of [ring-^{3}H]tyrosine was calculated as being half since a tritium atom in the 3- or 6-position was liberated in the reaction pathway of L-[3,5-^{3}H]tyrosine or L-[2,6-^{3}H]tyrosine.

Another aliquot was used to determine the amount of $^{14}CO_2$ liberated through the formation of 5,6-dihydroxyindole. One milliliter of the reaction mixture was pipetted into the outer section of a Conway diffusion unit[5] which had an inner section containing about 1 g of barium hydroxide powder to absorb the $^{14}CO_2$. Next, 0.2 ml of 0.5 M sulfuric acid was added to the mixture to liberate the dissolved $^{14}CO_2$. The Conway unit was immediately closed with a glass plate and left standing for at least 40 min until the liberated $^{14}CO_2$ had been completely absorbed by the barium hydroxide. The radioactivity remaining in the outer solution (0.5 ml) was counted in the assay system of double labeling compounds (^{3}HQ/^{14}CQ) with a liquid scintillation counter. The amount of $^{14}CO_2$ evolved was estimated by subtracting the ^{14}C radioactivity of each sample from that of the blank.

Colorimetric Tyrosinase Assay——Dopachrome was determined by recording absorbance changes at 475 nm (ε=3700) at 37°C using nonradioactive tyrosine with a Shimadzu recording spectrophotometer, Modei UV-200.

Distribution of Tritium in L-[3,5-^{3}H]Tyrosine and L-[2,6-^{3}H]Tyrosine——The distribution patterns of tritium in a tracer for hydrogen must be precisely known. The distribution of [ring-^{3}H]tyrosine was checked by following the enzymatic dopa formation from L-[carboxyl-^{14}C]tyrosine. Tyrosine hydroxylation by tyrosinase was carried out at 37°C with an incubation mixture of 10 mM L-tyrosine containing either L-[3,5-^{3}H]tyrosine or L-[2,6-^{3}H]tyrosine (1 μCi) together with L-[carboxyl-^{14}C]tyrosine (0.1 μCi), 2000 units of tyrosinase and 25 mM phosphate buffer, pH 6.8, in a total volume of 1.0 ml. The reaction was stopped with 0.2 ml of 6 N HCl after incubation for 5 min, and 0.1 ml of 10 mM L-dopa was added as the carrier of radioactive dopa. One milliliter aliquots were placed on a 0.9 × 32 cm column of Dowex 50 W × 2 (H^{+} form, 200—400 mesh) and eluted with 3 N HCl. Dopa and tyrosine were eluted at 70—90 and 110—125 ml, respectively, and their radioactivity levels were measured with a liquid scintillation counter.

In the reaction solution of L-[3,5-^{3}H]tyrosine and L-[carboxyl-^{14}C]tyrosine, the ratio, ^{3}H/^{14}C, in the isolated dopa was 0.48 as compared to 1.0 in the initial tyrosine. L-[3,5-^{3}H]Tyrosine has shown little or no tritium isotope effect in tyrosine hydroxylation.[6] Therefore, the ratio of 0.48 in the isolated dopa indicates that 96% of the label in L-[3,5-^{3}H]tyrosine must be equally distributed at the 3- and 5-positions. In the hydroxylation of L-[2,6-^{3}H]tyrosine and L-[carboxyl-^{14}C]tyrosine, the ^{3}H/^{14}C ratio in the isolated dopa was 0.916 to that of the initial tyrosine. Thus, 91.6% of the tritium must exist equally distributed at the 2- and 6-positions. The radioactivity levels of L-[3,5-^{3}H]tyrosine and L-[2,6-^{3}H]tyrosine were corrected using these labeling ratios.

Results and Discussion

Modification of the ^3H_2O Method

The sensitivity of the tyrosinase assay for tritiated tyrosine depends on the blank radioactivity and the recovery of ^3H_2O formed by the tyrosinase reaction. The methods of Nagatsu *et al.*[3] and Pomerantz[4] give significantly high blank levels and the lower limit of the detection of the latter is 0.8 nmol. Charcoal (Norit A) has been used to lower the blank levels by adsorbing the radioactive substrates and intermediates. However, this addition of charcoal must not affect the radioactivity of ^3H_2O. As shown in Table I, effective amounts of Norit A were investigated and the adsorption of ^3H_2O onto Norit A was found to be negligible until the addition of 300 mg to 5 ml of ^3H_2O. When the blank reaction mixture was treated with 300 mg of Norit A, the remaining radioactivity of the filtrate decreased to 1% of the initial radioactivity. The filtrate was passed through a Dowex 50W×8 (H^+ form, 50—100 mesh) column (1.5 cm×3.0 cm) and tritiated water was thoroughly isolated from the remaining substrate and intermediates. As tritiated water was eluted at 4 to 9 ml, the eluate was collected to make a total of 10 ml. When the radioactive blank mixture was incubated for 40 min and treated under these conditions, ^3H radioactivity of 1 ml of the eluate did not exceed 50 cpm (efficiency with respect to ^3H: 34%). Therefore, in this method, if 1 ml of eluate has a radioactivity of at least 100 cpm, intermediates can be determined at the level of 0.1 pmol with reasonable accuracy.

Table I. Adsorption of ^3H_2O with Various Amounts of Norit A

Norit A (mg)	Adsorption of ^3H_2O (%)
100	0
200	0
300	0
400	0.7
500	2.3

Norit A was added to 5 ml of ^3H_2O (1 μCi/ml). The adsorption of ^3H_2O was estimated from the ratio, no Norit A−Norit A/no Norit A, of the radioactivity of the filtrate.

Apparatus for the $^{14}CO_2$-Release Method

The determination of the amount of $^{14}CO_2$ liberated through the formation of 5,6-dihydroxyindole from L-[carboxyl-^{14}C]tyrosine was initially carried out in a closed Warburg vessel with a single sidearm and a center well containing 0.2 ml of either monoethanolamine or 20% KOH as the absorbent of $^{14}CO_2$. However, at most only 80% of the $^{14}CO_2$ evolved was trapped even after sulfuric acid had been added to the reaction mixture to thoroughly liberate any dissolved $^{14}CO_2$. The radioactivity of the reaction mixture decreased with liberation of $^{14}CO_2$ and the formation of 5,6-dihydroxyindole could be estimated from the remaining radioactivity. However, the remaining radioactivity must be free from that of any dissolved $^{14}CO_2$. A Conway diffusion unit (inner section, $Ba(OH)_2$; outer section, $Na_2{}^{14}CO_3$ solution) was treated with a $Na_2{}^{14}CO_3$ standard (Table II). As the remaining radioactivity of the outer section decreased to 0.12% at 40 min after the addition of sulfuric acid, the standing time was set at 40 min. To verify that the $^{14}CO_2$-release method with the Conway unit gives valid data for the carboxylation step, the ^3H_2O method was undertaken with L-[side chain -2,3-^{3}H]tyrosine. At the decarboxylation step, the amount of $^{14}CO_2$ liberated from L-[carboxyl-^{14}C]tyrosine is equal to that of the ^3H_2O released from L-[side chain-2,3-^{3}H]tyrosine. The reaction mixture containing both labelled tyrosines was prepared and assayed by the ^3H_2O and the $^{14}CO_2$-release

methods. The radioactivity of each of the two hydrogens in the β-position of L-[side chain-2,3-^{3}H]tyrosine is 25% since only one of them is labelled. The radioactivity of ^3H_2O released was corrected for the labelling ratio. As shown in Fig. 1, the amounts of $^{14}CO_2$ determined by the $^{14}CO_2$-release method agreed closely with those of ^3H_2O determined by the ^3H_2O method. Therefore, the $^{14}CO_2$-release method with the Conway diffusion units could be used to measure 5,6-dihydroxyindole formation and permitted the detection of as little as 0.1 pmol of 5,6-dihydroxyindole.

Estimation of the Maximum Velocity of the Tyrosinase Reaction

In the reaction pathway from tyrosine to melanin catalyzed by tyrosinase, the formations

TABLE II. Variation of the Radioactivity Remaining in the $Na_2{}^{14}CO_3$ Solution after the Addition of Sulfuric Acid

Standing time (min)	Radioactivity remaining in the $Na_2{}^{14}CO_3$ solution (%)
20	0.61
40	0.12
120	0.09

One ml of 50 mM $Na_2{}^{14}CO_3$ (0.1 μCi) and 1 g of barium hydroxide powder were put in the outer and inner section of a Conway diffusion unit, respectively, and then 0.2 ml of 0.5 M sulfuric acid was added to the $Na_2{}^{14}CO_3$ solution.

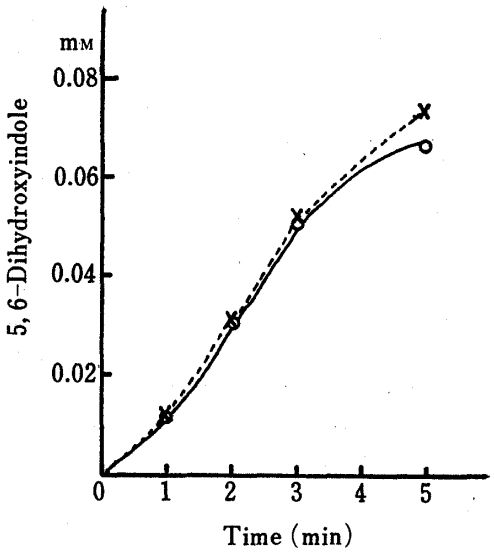

Fig. 1. Formation of 5,6-Dihydroxyindole from L-[Side chain-2,3-^{3}H]tyrosine and L-[Carboxyl-^{14}C]tyrosine by Tyrosinase

The reaction mixture consisted of 0.5 mM tyrosine containing ^{3}H-tyrosine (1 μCi, ○) and ^{14}C-tyrosine (0.1 μCi, ×), 100 units of tyrosinase and 25 mM phosphate, pH 6.8, in a volume of 1 ml.

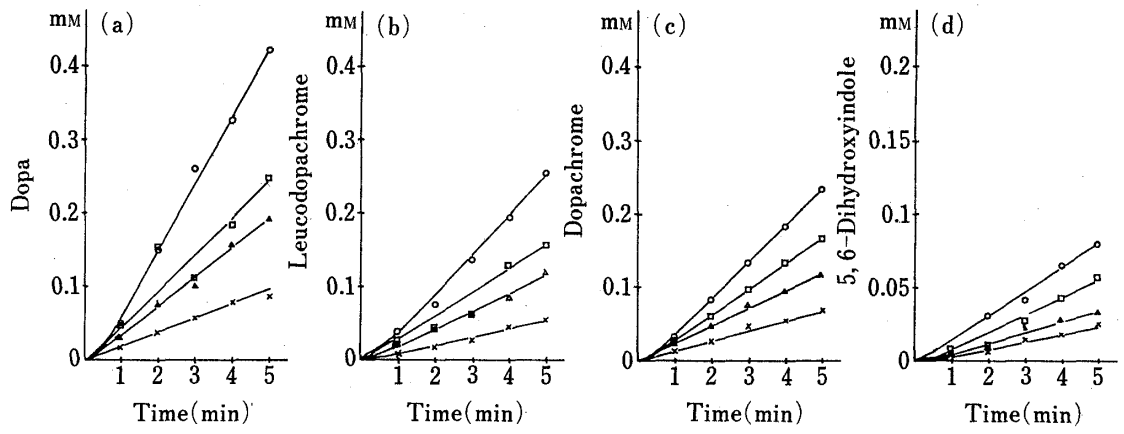

Fig. 2. Formation of Dopa (a), Leucodopachrome (b), Dopachrome (c), and 5,6-Dihydroxyindole (d) from Tyrosine by Tyrosinase

The reaction mixture contained tyrosine (0.8 mM, ○; 0.4 mM, □; 0.2 mM, △; 0.1 mM, ×) and 100 units of tyrosinase in 1 ml of 25 mM phosphate buffer, pH 6.8. The radioactive substrates added were: (a) L-[3,5-^{3}H]tyrosine, (b) L-[2,6-^{3}H]tyrosine, (d) L-[carboxyl-^{14}C]tyrosine.

of L-dopa, leucodopachrome and 5,6-dihydroxyindole were assayed by the $^{3}H_2O$ method and the $^{14}CO_2$-release method, and dopachrome formation was measured colorimetrically. Figure 2 shows the time courses of formation of L-dopa, leucodopachrome, dopachrome and 5,6-dihydroxyindole at various concentrations of tyrosine. From these results, Lineweaver–Burk plots of the inverse of the initial reaction rate *versus* the inverse of the L-tyrosine concentration were drawn and the maximum velocity of each rate was estimated (Table III). The radiochemical tyrosinase assay using L-[ring-^{3}H]tyrosine and L-[carboxyl-^{14}C]tyrosine permitted the simultaneous determination of two types of intermediates produced through the oxidation of tyrosine. It should help to clarify the reaction pathway in the synthesis of melanin from tyrosine catalyzed by various tyrosinases.

Table III. Comparison of the Maximum Velocities of Formation of Dopa, Leucodopachrome, Dopachrome and 5,6-Dihydroxyindole from Tyrosine by Mushroom Tyrosinase

Experiment number	Maximum velocity (10^{-2} mM/min)			
	Dopa	Leucodopachrome	Dopachrome	5,6-Dihydroxyindole
1	12.5	—	7.7	2.6
2	11.8	—	9.1	3.8
3	14.3	—	9.1	3.6
4	16.1	—	7.1	3.5
5	14.3	—	9.1	3.6
6	16.7	—	9.1	3.6
7	—	12.5	9.1	3.6
8	—	8.3	7.1	2.6
9	—	10.0	7.7	2.8
10	—	10.0	7.0	3.2
11	—	9.5	9.1	3.6
Average ±S.D.	14.3 ±1.8	10.1 ±1.4	8.3 ±0.9	3.3 ±0.4

References

1) *a*) H.S. Mason, "Pigment Cell Biology," ed. by M. Gordon, Academic Press, New York, 1959, pp. 563—582; *b*) M.S. Blois, *Photochem. Photobiol. Rev.*, **3**, 115 (1978); *c*) V.J. Hesring, Jr., T.M. Ekel, P.M. Montague, and J.M. Nicholson, *Biochem. Biophys. Acta*, **611**, 251 (1980).
2) S.H. Pomerantz, *J. Biol. Chem.*, **238**, 2351 (1963).
3) T. Nagatsu, M. Levitt, and S. Udenfriend, *Anal. Biochem.*, **9**, 122 (1964).
4) S.H. Pomerantz, *Anal. Biochem.*, **75**, 86 (1976).
5) E.J. Conway, "Microdiffusion Analysis and Volumetric Error," Crosby, Lockwood and Son, Ltd., London, 1939, p. 176.
6) S.H. Pomerantz, *J. Biol. Chem.*, **241**, 161 (1966).