IDENTIFICATION OF 3,4-DIHYDROXYPHENYLALANINE AS TYROSINASE COFACTOR IN MELANOMA*

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The tyrosine hydroxylase activity of tyrosinase from hamster melanoma requires a reduced co-substrate. The most efficient co-substrate for in vitro experiments was shown to be L-3,4-dihydroxyphenylalanine (dopa) (Pomerantz, 1964, 1966); only a catalytic amount of dopa was required because it is also a product of the hydroxylation (Equation (1)). The cofactor activity of dopa

$$\text{L-tyrosine-3,5-}^{3}\text{H} + \text{L-dopa} + O_2 \longrightarrow \text{L-dopa-5-}^{3}\text{H} + \text{L-dopa quinone} + {}^{3}\text{HOH} \quad (1)$$

for tyrosinase may be contrasted with the reduced pteridine required for the tyrosine hydroxylase of adrenal medulla (Brenneman and Kaufman, 1964; Nagatsu et al, 1964).

This report describes the concentration from hamster melanoma of a substance with cofactor activity for tyrosine hydroxylation by tyrosinase, and the identification of this substance as L-dopa.

Materials and Methods: L-tyrosine decarboxylase from S. fecalis was purchased from Sigma; N-methyl-N-nitroso-p-toluenesulfonamide from Aldrich; N-methyl-^{14}C-N-nitroso-p-toluenesulfonamide from New England Nuclear; and L-dopa 2,5,6-^{3}H from Nuclear Chicago. The origins of the 250-fold purified hamster melanoma tyrosinase and other materials have been given previously (Pomerantz, 1963, 1966).

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Hydroxylation was estimated by counting an aliquot of the ^{3}HOH produced from L-tyrosine-3,5-^{3}H in the incubation mixture described earlier (Pomerantz, 1966). The dopa-catalyzed reaction was run for 20 minutes with the addition of 0.15 μmole dopa, an amount which yields a linear rate of hydroxylation. To correct for the inhibitory effects of crude tissue fractions, these were added to standard dopa incubations.

The assay for cofactor activity was based on the reduction by melanoma extracts of the lag preceding tyrosine hydroxylation in the absence of dopa during a 60-minute incubation. One unit of cofactor activity is defined as the quantity permitting 3 times the amount of ^{3}HOH as that produced in 20 minutes from the standard dopa-catalyzed reaction. In a given time period and over a limited range of concentrations of the test substance, the extent of

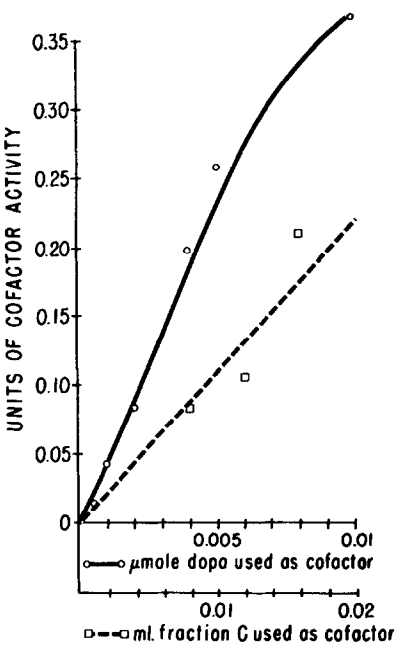

Fig. 1 Relationship between dopa or Fraction of tumor extract and units of cofactor activity.

Each point represents one 60-minute reaction using the amount of dopa or the volume of Fraction C indicated and the assay system described in the text. The extent of each hydroxylation was converted into units by comparison with a reaction catalyzed by 0.15 μmole dopa, as given in the text.

hydroxylation was directly proportional to the amount of cofactor added, as shown in Fig. 1. The cofactor response with a sub-optimal amount of dopa was also linear with the quantity of dopa, to about 0.005 μmole. In the 60-minute assay, one unit of cofactor is equivalent to 0.02 μmole dopa.

Concentration of Cofactor Activity: The method summarized is the procedure currently in use. Hamster melanoma (400 g.) was homogenized in the cold in 0.01 M phosphate, pH 6.5, made 5% in $HClO_4$, and the mixture was centrifuged. The supernatant was titrated with KOH to pH 7.9 and filtered from the precipitate of $KClO_4$. The filtrate (Fraction A) was made 0.1 N in HCl and applied at room temperature to a 1.9 x 20 cm. column of Dowex 50 (H^+). The column was eluted successively with 0.1 N HCl, 1.0 N HCl, 2.0 N HCl, and 4.0 N HCl. The last fraction, containing the activity, was concentrated by lyophilization (Fraction B), then mixed with 40 ml of 0.1 M sodium borate, pH 8.6, brought to pH 8.5 by the addition of 1 N NaOH, and then applied at room temperature to a 1.9 x 30 cm. column of Dowex 2 (borate) x 8, 100-200 mesh (Takahashi and Fitzpatrick, 1964). The column was eluted with 0.1 M sodium borate, pH 8.6, 2.5% boric acid, and 0.2 N HCl. The last fraction, containing active material, was applied to a 0.9 x 50 cm. column of Dowex 50 (H^+) x 8, 200-400 mesh. The column was washed with 1 N HCl and then eluted with a linear gradient of HCl (1 N $\longrightarrow$ 4 N) with a mixing chamber of 250 ml of 1 N HCl. The activity was found in tubes eluted with about 2 N HCl. Those tubes were concentrated (Fraction C) and applied to Whatman 3MM paper for descending chromatography in n-butanol: acetic acid: H_2O (80:20:20). The whole paper was cut into 1 cm. strips and portions of each were tested for activity. The appropriate strips were then eluted with 0.01 N HCl and concentrated (Fraction D). Table I shows the activities obtained in a typical preparation. The amino acid content of each fraction is used as an index of the purification achieved. The total activity of Fraction A in the last seven preparations ranged from 363 to 840 units.

Table I
Scheme for Purification of Cofactor for Tyrosinase

Fraction	Volume ml	total units	Total amino acids* μmole	Units/μmole of amino acid	Yield %
A	1420	363	6820	0.052	100
B	10	233	1240	0.19	64
C	5	58	12.1	4.8	16
D	3	32	1.62	20	8.8

*Determined by ninhydrin analysis
Duplicate or serial aliquots from each fraction were assayed as indicated in the text and the average values are reported.

Identification of Dopa in Active Fractions: Several lines of evidence point to dopa as the compound responsible for the activity:

(1) Co-electrophoresis of active material with dopa-2-^{14}C at pH 4 (pyridine-acetic acid) and pH 1.9 (formic-acetic acids) and co-chromatography in three solvent systems. In each case DL-dopa-2-^{14}C (2400 cpm, 0.001 μmole, 0.05 unit of cofactor activity) was mixed with 0.9 - 1.5 units of cofactor containing from 4-6 μmoles of amino acids (from a purification scheme different from Table I). The chromatographic systems were tert-butanol: formic acid: water (70:15:15), methyl ethyl ketone: propionic acid: water (60:20:24), and n-butanol: acetic acid: water (4:1:1). In each case there was a coincidence of dopa-^{14}C and cofactor activity.

(2) When Fraction D was treated with mammalian tyrosinase the absorption spectrum of the pink reaction mixture resembled that derived from dopa, with a broad maximum between 460 and 500 mμ. The rate of formation of material absorbing at 475 mμ, from 1.9 units, was about the same as the rate of dopachrome formation from 0.025 μmole of dopa (1.2 units).

(3) Table II shows another comparison of cofactor activity with dopa beyond the range of linear response: 6.2 units of Fraction D was equivalent in response to 0.10 μmole dopa (5 units) in nearly eliminating the lag.

(4) Methylation of Fraction D with $^{14}CH_2N_2$ gave a derivative which migrated on electrophoresis at pH 1.9 with purified 0,0'-dimethyldopa methyl ester, formed by treatment of dopa with CH_2N_2 (Figure 2). The area corresponding to the cofactor derivative was cut out, eluted, and chromatographed in methyl ethyl

Table II
Comparison of Cofactor Activity with Dopa in Eliminating Lag During 20-Minute Assay

Cofactor	Units	Equiv. amt. dopa* μmole	^{3}HOH in aliquot dpm
Fraction D	6.2	0.12	18,700
Dopa, 0.05 μmole	2.5*	-	13,700
0.10 μmole	5.0*	-	18,800
0.15 μmole**	7.5*	-	22,200

* Determined from approximate relationship of 1 unit as the equivalent of 0.02 μmole dopa.

**This amount eliminates the lag completely.

The reaction mixture contained, besides Fraction D or dopa as shown, L-tyrosine-3,5-^{3}H (1 μmole, 5-7x10^6 dpm); phosphate buffer, pH 6.8 (35 μmole); enzyme (1.2 units); 1.25 ml. volume. The incubation at 37° was stopped after 20 min. ^{3}HOH was isolated and counted as described (Pomerantz, 1966).

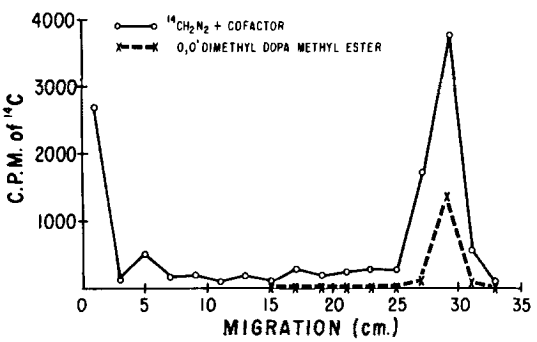

Fig. 2. Distribution of radioactivity after electrophoresis of crude mixture from the treatment of Fraction D with $^{14}CH_2N_2$.

Diazomethane-^{14}C, generated from N-methyl-^{14}C-N-nitroso-p-toluenesulfonamide (50 μmoles, 6x10^5 dpm/μmole) (Vogel, 1957), was passed into an ethanolic solution of Fraction D (7.8 units). The solution was evaporated in a stream of air, dissolved in a small volume of alcohol, and subjected to electrophoresis at pH 1.9 (formic-acetic acids) for 2 hours at 4000 volts and 25-30 mamp. A purified sample of O,O'-dimethyldopa methyl ester, prepared from dopa-2,5,6-^{3}H and $^{14}CH_2N_2$, was run on the same sheet. The methylated dopa had been identified by comparison of the ratio of ^{3}H to ^{14}C in the product with the specific activities of dopa-2,5,6-^{3}H and $^{14}CH_2N_2$. The crude dopa reaction mixture also contained a methylated product which remained at the origin.

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ketone: propionic acid: water (60:20:24). Again the cofactor derivative migrated to the same area as the dopa derivative.

(5) Incubation of Fraction D (1.4 units) with tyrosine decarboxylase led to a product which corresponded to dopamine by electrophoresis at pH 1.9. Fig. 3 shows the distribution of counts on an electrophoresis sheet after the re-

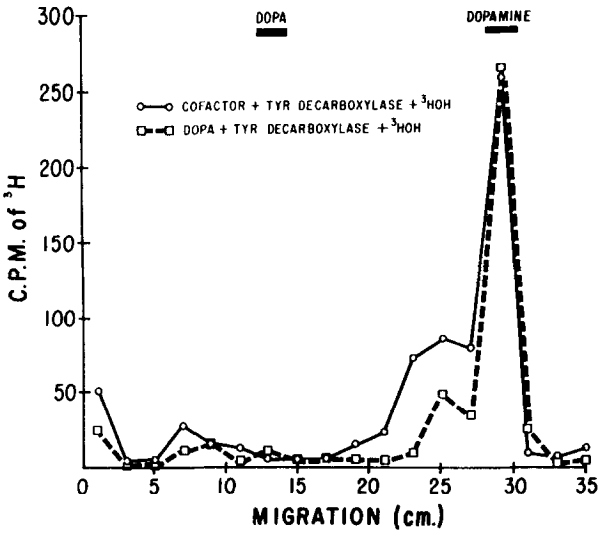

Fig. 3. Distribution of radioactivity from electrophoresis of crude reaction mixture of either Fraction D or dopa and tyrosine decarboxylase.

Fraction D (9.0 units) or dopa (0.20 μmole) was incubated at 37° for 30 minutes with 2 mg. tyrosine decarboxylase in 0.32 ml of solution containing 10 μmole sodium citrate buffer, pH 5.5 and 2.5 x 10^5 dpm/μmole of ^{3}HOH. After stopping with 1N HCl (0.10 ml), the solutions were centrifuged and the supernatants lyophilized and subjected to electrophoresis at pH 1.9 as described under Fig. 2. Dopa and dopamine were spotted alongside as markers.

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action was carried out in ^{3}HOH, a procedure designed to introduce ^{3}H into the expected product (Mandeles et al, 1954). Coincidence with the ^{3}H peak from the decarboxylation of dopa is also apparent.

Discussion: The evidence establishes dopa as present in Fraction D, and from the correlation of units of activity and μmoles of dopa (points (2) and (3) above), it is probable that dopa accounts for all the activity in that fraction. Because of the large loss in activity during purification it is possible that other active components may have been present in an early fraction. However, after adding DL-dopa-2-^{14}C to a perchlorate-treated homogenate, the recovery of counts in Fraction B was 74% of that found in Fraction A and only 50% of that added to the homogenate. Consequently the large losses observed are compatible with losses encountered with dopa itself.

If dopa accounts for all the activity in the tumor, its concentration is

about 2-4 μmole/100 g. or 4-8 μg/g. Anton and Sayre (1964) were unable to detect any dopa in a number of tissues from several animals, although they did find large quantities in the urine of neuroblastoma patients. Cabana et al (1964) reported dopa in the media of pheochromocytoma grown in tissue culture. Recently Takahashi and Fitzpatrick (1966) reported large quantities of dopa (230 μg/g.) in acid hydrolysates of the trichloroacetic acid (TCA) precipitate of Harding-Passey mouse melanoma, but only 1 μg/g. in the TCA supernatant fraction. Our own assay of a hydrolysate of hamster melanoma showed only 64 units of cofactor/100g or about 2.6 μg dopa/g tumor, less than is usually found in the perchlorate supernatant. The significance of large quantities of dopa in the hydrolysate of Harding-Passey melanoma remains to be determined.

The presence of dopa in a tissue which contains tyrosinase is suggestive that dopa is the natural cofactor for the enzyme. If dopa is both required and synthesized by tyrosinase it is possible that an independent route to the synthesis of dopa occurs in melanoma, perhaps via tyrosine hydroxylase.

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