A SENSITIVE FLUOROMETRIC ASSAY METHOD FOR MAMMALIAN TYROSINASE*

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Tyrosinase in the mammalian melanocyte catalyzes the initial steps in melanogenesis, i.e., the hydroxylation of tyrosine to dihydroxyphenylalanine (DOPA) and the subsequent oxidation of DOPA to DOPA quinone. The kinetic characteristics of the hydroxylation step had been difficult to study until Pomerantz (1964, 1966) developed a specific and sensitive assay method based on the measurement of the release of tritium from tyrosine-3,5-^{3}H. The method requires a column chromatography step and the use of radioisotopes. Therefore it is not well suited to the handling of large numbers of biological samples. In this paper, we report an adaptation of the fluorometric measurement of DOPA (Bertler et al., 1958) as applied to the assay of mouse melanoma tyrosinase (tyrosine hydroxylase). With this method microgram quantities of tissue with an active tyrosinase system can be used. The method is quite applicable to the study of the changes in enzyme activity with increasing age of melanomas (Adachi, unpublished observations) and probably also to all melanizing tissues.

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MATERIALS AND METHODS

The complete reaction mixture for the enzyme assay consisted of: 0.75 mM L-tyrosine; 100 μM L-(or DL-) DOPA; 0.75 mM ascorbic acid; 40 mM phosphate buffer, pH 6.9; and 25 μl of the enzyme in a total volume of 50 μl. The ascorbic acid and DOPA solutions were prepared fresh each day.

Fresh melanoma samples (B16 mouse melanoma transplanted successively in C 57 BL mice*) weighing 50-500 mg were homogenized at a concentration of 50% (W/V) in water at 2° C with a ground glass homogenizer. The homogenate was centrifuged at 3,000 rpm for 10 minutes and the resultant supernatant fraction was used as enzyme.

Incubations at 37° C were carried out for time periods of up to 60 minutes. The reaction was stopped by immersing the tubes in ice water and the DOPA formed during the incubation period was measured in triplicate.

The procedure for the development and measurement of the DOPA fluorescence was carried out at room temperature. Five microliters of the mixture were transferred into 900 μl of 10 mM pH 6.5 phosphate buffer containing 0.0025% zinc sulfate solution in a 3 ml fluorometer tube. After mixing, 10 μl of 0.25% potassium ferricyanide were added and the contents of the tube were mixed immediately (oxidation step). After exactly 2 minutes (this duration of oxidation is critical), the oxidation was stopped by the addition of 100 μl of a mixture of 5N NaOH and 2% ascorbic acid solution, 9:1 (V/V). Five minutes later the samples were read in a spectrofluorometer (excitation @ 360 mμ, fluorescence @ 490 mμ). Alternatively, an ordinary fluorometer may be used with commercially available Corning filters, No. 5860 as the primary (365 mμ), and Nos. 3385 and 4305 as the secondary (peak at 505 mμ, range from 480 to 580 mμ).

Blanks lacking both tyrosine and DOPA but containing enzyme were run

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* Melanoma samples were kindly supplied by Dr. Funan Hu, Oregon Regional Primate Research Center.

simultaneously. Standards consisted of 1) the complete reaction mixture containing boiled enzyme (100° C for 5 minutes) instead of native enzyme, and 2) the complete reaction mixture kept at 0° C for the same period of time as the actual samples.

The radiochemical assay, which was run for comparison with the fluorometric method, was based on a measurement of released tritiated water from L-tyrosine-3, 5-^{3}H by tyrosinase according to the method of Pomerantz. The radiochemicals were obtained through New England Nuclear Corporation. Protein was determined with the Folin-Ciocalteau reagent (Lowry et al., 1951).

RESULTS AND DISCUSSION

DOPA in the incubation mixture is necessary to eliminate the initial lag period which is found when only the substrate tyrosine and enzyme are present (Figure 1). In agreement with Pomerantz (1964, 1966) we found that the catalytic amount of DOPA needed to eliminate this initial lag in tyrosine hydroxylation varied with different samples, but generally speaking 100 μM DOPA is sufficient to completely inhibit the lag phase and this amount of DOPA was used in the routine assays. Ascorbic acid did not eliminate the lag at any concentration but concentrations higher than 10 mM clearly inhibited

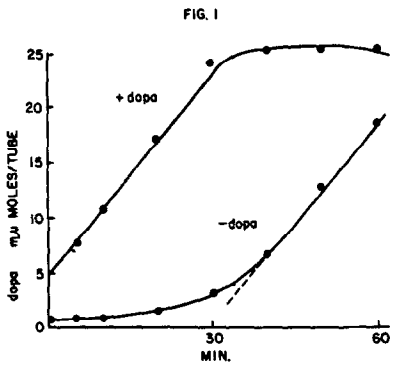

Figure 1 Effect of DOPA (100 μM) on the initial lag period. Test system as in the text.

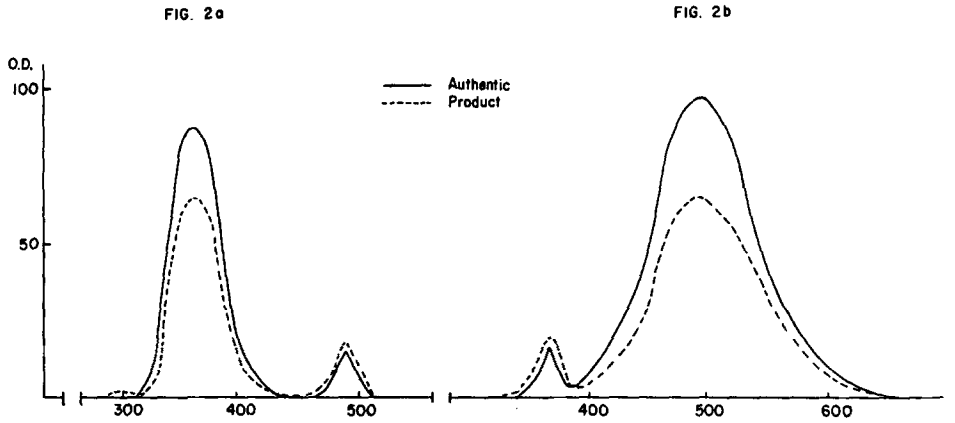

Figure 2 Fluorescence spectra of reaction product compared with authentic DOPA
a) emission constant @ 490 mμ +DOPA not present in reaction tubes
b) excitation constant @ 360 mμ

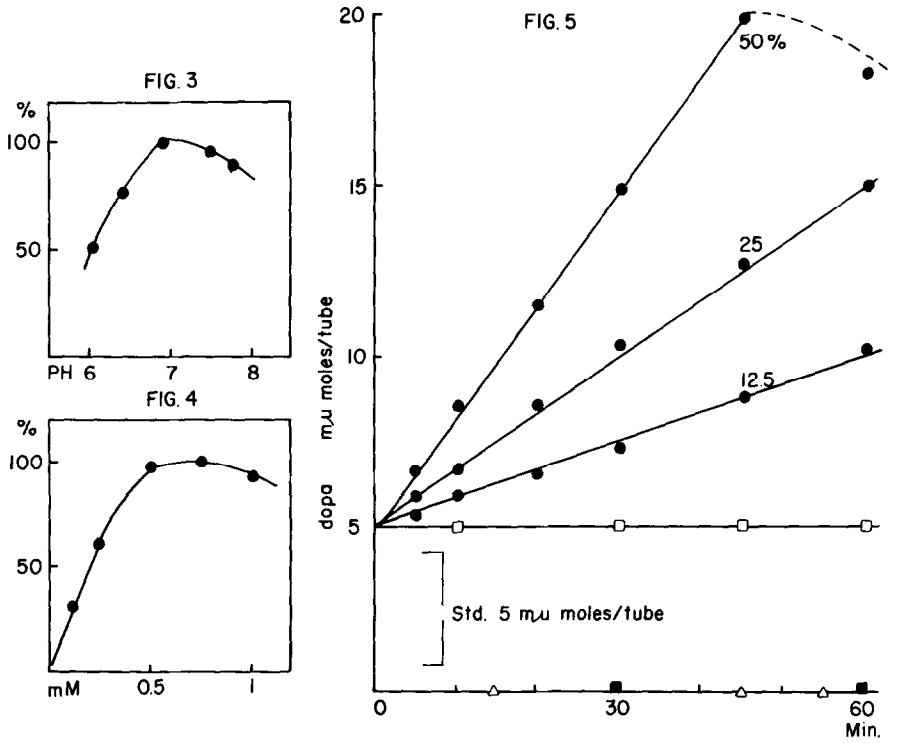

Figure 3 Effect of pH on enzyme activity. Enzyme activities are shown as % of maximal rate of DOPA formation.

Figure 4 Effect of tyrosine concentration on enzyme activity.

Figure 5 Time course of the reaction with different amounts of enzyme. △ .....blank; □ .....standard as described in the method.

development of fluoroescence at the oxidation step with potassium ferricyanide.

Mouse melanoma tyrosinase activity is strongly dependent on temperature. The rate of the reaction at room temperature (22.5° C) was only 17% of that at 37° C, and no reaction occurred at 2° C. Therefore, the reaction mixtures kept in ice water may be used as standards. Figure 2 demonstrates that the reaction product formed from tyrosine is actually DOPA as shown by their excitation and emission fluorescence spectra.

FIG. 6

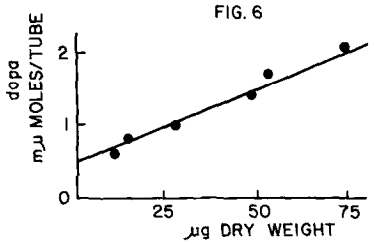

Figure 6 Enzyme activity in frozen dried microgram samples. Frozen dried melanoma samples were incubated for 30 minutes with the final reagent mixture of 5 μl. For detailed instrumentations and techniques of micro-assay system, see Lowry, 1953.

Figure 3 shows maximal activity of mouse tyrosinase at approximately pH 6.9 in phosphate buffer. Figure 4 demonstrates the effect of substrate concentration on observed activity. The apparent Km for tyrosine is 6×10^{-4} M and concentrations exceeding 0.75 mM are slightly inhibitory. Figure 5 illustrates the time course of the reaction with different amounts of enzyme. Linear reaction rates were observed up to 20 mμmoles of DOPA production/tube. Activities during this period are directly proportional to the amount of enzyme added.

A comparison of assays done by both the fluorometric and the radiochemical method is shown in Table I. By both methods mouse melanoma tyrosinase activities vary from 1.0 - 3.8 mmoles of tyrosine hydroxylated/hr/kg fresh tissue. As seen from Table I, agreement between the two methods is good.

Since an increase in DOPA concentration equal to the original catalytic amount present in the reaction vessel (0.1 mumole/λ) can easily be determined,

Table I

Comparison of the rates of tyrosine hydroxylation* measured by isotopical and fluorescent methods

Tyrosine hydroxylation	Determined	
	as ^{3}H-water released	as dopa fluorescence formed
	Ave. ± S.E. (Range)	Ave. ± S.E. (Range)
Sample 1	2.6 ± .32 (2.0-3.8)	2.3 ± .08 (2.2-2.6)
Sample 2	1.2 ± .09 (1.0-1.5)	1.3 ± .05 (1.2-1.5)

* Expressed as mmoles of tyrosine hydroxylated per hour per kg wet weight. Average of 5 determinations.

the sensitivity of the method can be increased by scaling down the reaction volume to 5 µl instead of 50 µl. The formation of 0.5 mµmoles of DOPA can then be detected and it is possible to use as little as 10-50 µg of dried melanoma tissue for the assay (Figure 6). With tissues containing lesser amounts of tyrosinase activity more tissue is of course necessary, e.g., rhesus monkey retina is only 1/5 as active as mouse melanoma.

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