TYROSINE HYDROXYLATION CATALYZED BY MAMMALIAN TYROSINASE: AN IMPROVED METHOD OF ASSAY*

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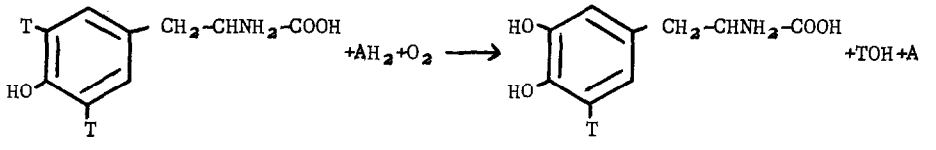
Tyrosinases, classed as mixed-function oxygenases (Mason, 1957), catalyze the hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanine (dopa) and the oxidation of dopa to melanin via several intermediates:

L-tyrosine ⟶ L-dopa ⟶ dopa quinone ⟶ ⟶ dopachrome ⟶ ⟶ melanin

A lag, observed in the tyrosine reaction when measured by oxygen uptake or dopachrome formation, can be shortened, but usually not eliminated, by catalytic amounts of dopa. Systematic comparison of compounds that shorten the lag, as well as other kinetic studies, have been hampered by inadequate methods for measuring tyrosine hydroxylation. Thus, assay of dopa formation (Kendal, 1949) requires a high concentration of ascorbate to reduce dopa quinone to dopa non-enzymatically, while the measurement of tyrosine disappearance (Krueger, 1950) is subject to large errors early in the reaction.

This paper reports the use of L-tyrosine-3,5-T for studying the initial step catalyzed by tyrosinase:



The rate of tritium released as water is directly proportional to the rate of hydroxylation. Different reducing agents can be readily compared

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and initial rates sensitively determined. The method was compared with the technique of using excess ascorbate to accumulate dopa.

These experiments indicate that (1) tyrosine hydroxylation by mammalian tyrosinase requires the presence of a hydrogen donor; (2) dopa is the most efficient reducing agent for this purpose, but a reduced pteridine, effective in phenylalanine hydroxylation (Kaufman, 1959) and with adrenal and brain tyrosine hydroxylase (Nagatsu et al., 1964a, b), is effective at higher concentration; (3) ascorbate, even at high concentration, does not eliminate the lag; (4) dopa can act as a competitive inhibitor of tyrosine hydroxylation; (5) tyrosine exhibits an apparent substrate inhibition at concentrations higher than about 8×10^{-4}M in the presence of catalytic quantities of dopa; (6) there is little or no tritium rate effect.

Materials and Methods: The enzyme was purified 250-fold from hamster melanoma (Pomerantz, 1963) (referred to as Enzyme I in the reference). L-Tyrosine-3,5-T and L-Tyrosine-U-C^{14} were purchased from New England Nuclear Corp. Since L-tyrosine-3,5-T is subject to radiation decomposition which leads to the release of some volatile product (probably water), samples were evaporated to dryness shortly before use. Norit A was furnished by Pfanstiehl, chromatographic grade aluminum oxide by Merck, and 2-amino-6,7-dimethyl-4-hydroxypteridine by Aldrich Chemical Co. The pteridine was reduced to the 5,6,7,8-tetrahydropteridine (ADHTP) with PtO_2 (Pohland et al., 1951).

Standard reaction mixtures contained, per tube, L-tyrosine-3,5-T, $10\text{-}20 \times 10^6$ dpm or L-tyrosine-U-C^{14},414,000 cpm, 1.0 μmole; L-dopa, 0.05-0.15 μmole; phosphate buffer, pH 6.8, 35 μmole; neutralized ascorbate when indicated, 14.8 μmole; enzyme, 1-7 units; volume, 1.25 ml; temperature, 37°. The reactions were stopped with metaphosphoric acid. Rates were obtained by assaying TOH, dopa-T, or dopa-C^{14} at five to seven time intervals and were corrected for counts obtained in boiled enzyme controls.

To count tritiated water the acidified reaction mixture was passed onto a 1.0 cm. column packed with a mixture of Norit A (100 mg.) and Celite 535 (500 mg). The column was washed with water to make an effluent volume of 25 ml. An aliquot (0.5 ml) was dissolved in 20 ml of dioxane scintillator fluid (Bray, 1960) and counted. Control sutdies showed that all the organically bound tritium was removed by the charcoal and that 25 ml was sufficient to remove all tritiated water.

When dopa-C^{14} or dopa-T was assayed, 2 μmoles of dopa were added to the acidified reaction mixture and it was passed through a 1 cm. column packed with 2 g. of aluminum oxide. Tyrosine was washed through with 0.5 M ammonium acetate and dopa was eluted with 0.5 N acetic acid. The dopa solution was acidified with dilute HCl, evaporated under reduced pressure, and purified by rechromatography on aluminum oxide. The purified dopa solution was assayed colorimetrically (Arnow, 1937) and counted. The known recovery was used to calculate the amount of dopa-C^{14} synthesized.

Results: To measure the stoichiometry L-tyrosine-3,5-T was incubated with ascorbate and one reaction tube at each time interval was separated on charcoal-celite while a companion tube was separated on aluminum oxide. The average rate of formation of TOH was 0.011 μmole/min. and that of dopa-5-T, 0.012 μmole/min.

The rate of formation of TOH in the presence of ascorbate, 0.0050 μmole/min./unit of enzyme (average of two experiments), was about the same as the rate in its absence, 0.0054 μmole/min./unit (average of four experiments). The rate of formation of dopa-C^{14} in the presence of ascorbate was 0.0058 μmole/min./unit. Hence there is little or no isotope rate effect in agreement with a finding of only a small tritium rate effect (Wood and Ingraham, 1962) in the mushroom tyrosinase-catalyzed hydroxylation of 3,4-dimethylphenol. Determination of the exact magnitute of an effect must await further experimentation.

The effect of catalytic amounts of dopa is shown in Fig. 1. An extended lag occured in the absence of dopa, whereas dopa at 1.2×10^{-4}M completely eliminated the lag. With another enzyme preparation a concentration of 4×10^{-5}M dopa was needed.

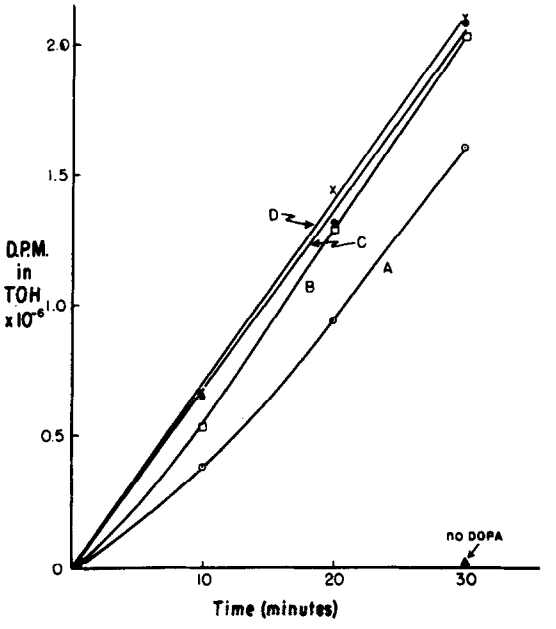

Fig. 1. Effect of various concentrations of L-dopa on the lag period in tyrosine hydroxylation.

The conditions are given in the text for L-tyrosine-3,5-T and 1.5 units of enzyme, except that the concentrations of dopa were varied as follows: Δ, no dopa; ⊙, 4×10^{-5}M (A); ⊡, 8×10^{-5}M (B); ●, 1.2×10^{-4}M (C); X, 1.6×10^{-4}M (D). No ascorbate was present.

Fig. 2 compares the effect of ADHTP at several concentrations with dopa at 4×10^{-5}M. ADHTP can substitute for dopa, but a higher concentration is needed to abolish the lag. Ascorbate, studied by the same method, does not eliminate the lag completely at 1.2×10^{-2}M. TPNH has a slight effect on the lag at 4.9×10^{-5}M, but DPNH at the same concentration has virtually no effect.

At higher concentrations dopa is a competitive inhibitor of tyrosine hydroxylation. This is shown by the plots in Fig. 3. The graph also re-

veals an apparent inhibition by tyrosine of its own hydroxylation at concentrations higher than about 8×10^{-4}M. The K_m for tyrosine is about 3×10^{-4}M by this method compared to 6×10^{-4}M found by dopachrome measurement (Pomerantz, 1963). The V_{max} is about 0.01 μmole of tyrosine converted per minute per unit of enzyme. The K_i for dopa (4×10^{-4}M) is similar to the K_m value of 5×10^{-4}M found earlier.

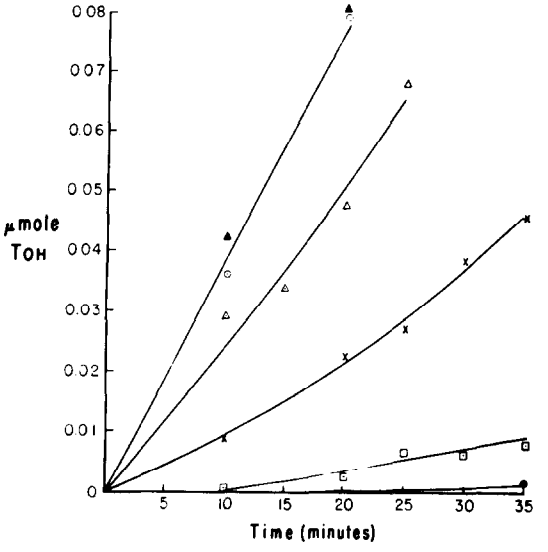

Fig. 2. 2-Amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine (ADHTP) as a hydrogen donor.

The conditions are given in the text for L-tyrosine-3,5-T and 0.96 units of enzyme, except that dopa (4×10^{-5}M) was used only with curve ⊙—⊙. ADHTP was used as follows: ▲——▲, 2.4×10^{-3}M; △——△, 8×10^{-4}M; X——X, 2.0×10^{-4}; ⊡——⊡, 4×10^{-5}M. No hydrogen donor with ——●. One curve is drawn for both dopa at 4×10^{-5}M and ADHTP at 2.4×10^{-3}M.

Discussion: This method establishes dopa as a competitive inhibitor of tyrosine hydroxylation and shows that tyrosine inhibits its own hydroxylation. It was found earlier that tyrosine is a competitive inhibitor of dopa oxidation (Pomerantz, 1963; Osaki, 1963). These results could be accomodated if excess tyrosine combined at the dopa site, as suggested by Osaki (1963) on kinetic grounds, thus preventing dopa from acting as a hydrogen donor, while excess dopa reacted with the tyrosine site, hence preventing tyrosine binding.

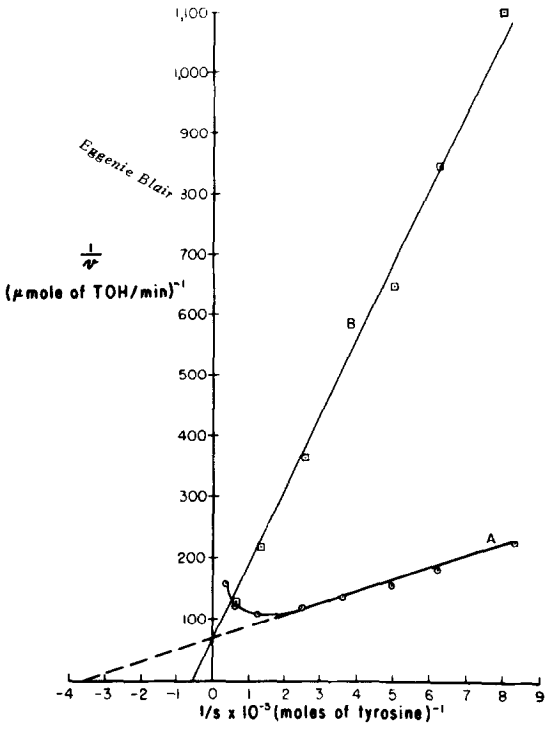

Fig. 3. Inhibition of tyrosine hydroxylation by dopa.

Each point represents the initial rate of reaction obtained by averaging rates found for 5,10, and 15 minute reaction times. The components used were given in the text except that tyrosine concentrations were varied as shown on the graph. Line A was obtained with catalytic amounts of dopa (1.2×10^{-4}M), while line B was obtained with an inhibitory quantity of dopa (2.4×10^{-3}M). No ascorbate present; 1.48 units of enzyme per tube.

Using crude mushroom tyrosinases Kendal (1949) found that high concentrations of ascorbate eliminated the lag in the formation of dopa and Krueger (1950) showed by tyrosine disappearance that ascorbate increased the reaction rate. Osaki (1963) reported that the initial oxygen uptake, measured by an oxygen electrode, occurred without a lag with highly purified mushroom tyrosinase in the absence of any hydrogen donor. These results are at variance with the data reported here for mammalian tyrosinase and might be caused by differences in enzyme specificity and source.

The reaction requires the presence of a substrate amount of reducing agent. However, only a catalytic amount of dopa is needed because it is

generated by the reaction. It is possible that ADHTP functions only to initiate the reaction, which then continues by utilization of the dopa produced.

ACKNOWLEDGEMENT

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References

Arnow, L. E., J. Biol. Chem., 118, 531 (1937).
Bray, G. A., Anal. Biochem., 1, 279 (1960).
Kaufman, S., J. Biol. Chem., 234, 2677 (1959).
Kendal, L. P., Biochem. J., 44, 442 (1949).
Krueger, R. C., J. Am. Chem. Soc., 72, 5582 (1950).
Mason, H. S., Adv. in Enzymology, 19, 79 (1957).
Nagatsu, T., Levitt, M., and Udenfriend, S., Biochem. Biophys. Res. Commun., 14, 543 (1964a).
Nagatsu, T., Levitt, M., and Udenfriend, S., Fed. Proc. 23, 480 (1964b).
Osaki, S., Arch. Biochem. Biophys., 100, 378 (1963).
Pohland, A., Flynn, E. H., Jones, R. G., and Shive, W., J. Am. Chem. Soc., 73, 3247 (1951).
Pomerantz, S. H., J. Biol. Chem., 238, 2351 (1963).
Wood, J. B. and Ingraham, L. L., Arch. Biochem. Biophys., 98, 479 (1962).