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Kinetics of Tyrosine Oxidation by Crude Tyrosinase Preparations from *Neurospora crassa*.* (23956)

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The ascomycete *Neurospora crassa*, strain 15300, is a mutant which differs from the wild-type at the albino-2 locus(1). Extracts of 15300 which contain the enzyme tyrosinase, are capable of oxidizing tyrosine, dopa (3-4 dihydroxyphenylalanine), or other mono- or dihydroxyphenols to corresponding 3-4 quinones, which are eventually converted to colored products (dopachrome in the case of tyrosine and dopa). We have for some time been engaged in an analysis of genetic control of synthesis and specificity of tyrosinase in strain 15300(2.3.4). The necessity to examine a large number of genetically different cultures has dictated the use of crude enzyme preparations and spectophotometric methods. This paper presents a study justifying the use of such methods and the use of crude extracts as a source of enzyme for determination of enzyme activity and specificity as reliable and dependable and not different from that of purified enzyme preparations.

Materials and methods. Large quantities of mycelium, from a single ascospore culture (15300-131a) isolated from the cross of 15300 A x 15300a, were obtained by growth on liquid minimal medium for 33 days at 25°C. After harvest, the mycelium was washed thoroughly and lyophilized. Enzyme preparations were made by homogenizing 23 mg of lyophilized mycelium in 2.50 ml of buffer (0.85% NaCl + 0.1 M phosphate pH 6.7) at 5°C for 5 minutes in an all-glass homogenizer. Aliquots of the supernate, collected after centrifugation at 5000 rpm for 20 minutes at 5°C, were tested for activity by measuring the rate of dopachrome formation spectrophotometrically, *i.e.* at 305 mμ or 475 mμ in a Beckman DU spectrophotometer. Reaction mixtures consisted of 0.25 ml of supernate + 3.25 ml of buffer containing defined amounts of l-tyrosine as substrate. Further details are provided elsewhere(4).

Results. Kinetics of dopachrome formation. The course of dopachrome formation in our reaction mixtures follows closely the course of tyrosine oxidation demonstrated by other workers using manometric methods and purified enzyme preparations (Fig. 1). There is a distinct induction period, followed by period of linearity, ultimately falling off as the reaction proceeds. Furthermore, the rate of dopachrome formation during the linear period is related to substrate concentration as expected on the basis of the Lineweaver-Burk modification of the Michaelis-Menten relationship (Fig. 2). This makes it possible to calculate both V_{max}, the reaction rate expected when substrate is unlimited, which measures enzyme concentration under these circumstances, and the apparent Michaelis Constant (K_s) of the enzyme. The rate of dopachrome formation is influenced by pH (Fig. 3), falling symmetrically on either side of pH 7.4, which can be taken as the pH optimum of the enzyme. All these observations strongly suggest that the spectrophotometric method is satisfactory for the measurement of tyrosine oxidation by crude tyrosinase preparations from *Neurospora*. A direct demonstration was nevertheless deemed important, and studies of the relationship of tyrosine utilization to dopachrome formation in our reaction mixtures were therefore undertaken.

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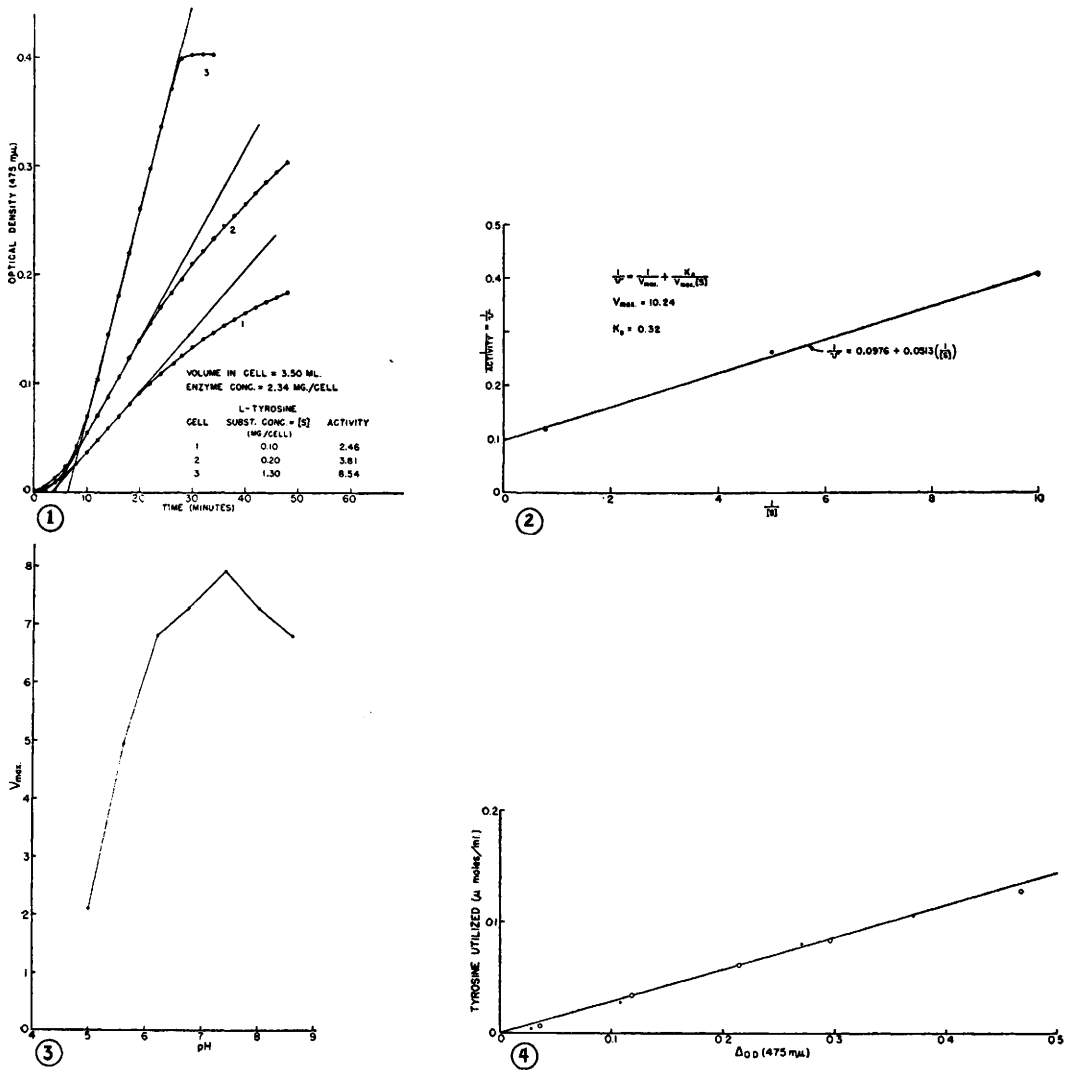


FIG. 1. Oxidation of tyrosine by extract of 15300-131*a* mycelium. Activities calculated from slopes of linear portions of respective curves = change of optical density/min./mg of lyophilized mycelium.

FIG. 2. Lineweaver-Burk kinetics of tyrosine oxidation by extract of 15300-131*a* mycelium. [S] = substrate conc. v = activity (see Fig. 1). $V_{max.}$ = reaction rate when substrate is unlimited. K_s = apparent Michaelis Constant.

FIG. 3. Effects of pH on tyrosinase activity of extract of 15300-131*a* mycelium. $V_{max.}$ calculated as in Fig. 2. Dopachrome production measured at 475 mμ.

FIG. 4. Relationship between tyrosine utilized and change in optical density during incubation of reaction mixture containing extract of 15300-131*a* mycelium. Open and solid circles represent results of 2 separate experiments.

Relationship of tyrosine utilization to dopachrome formation. Our task was to determine whether each mole of tyrosine utilized in the reaction mixture gives rise to one mole of dopachrome. Concentration of the latter was determined spectrophotometrically. Concentration of tyrosine was determined by means of a chromatographic method. Preliminary investigation showed that known quantities of tyrosine could be separated from amino acid, peptide, and protein mixtures by chromatography, detected on filter paper by complexing with ninhydrin, rendered light stable and methanol soluble by further complexing with

TABLE I. Relationship between Tyrosine Utilized and Dopachrome Produced by Reaction Mixtures Containing 15300–131*a* Extract.

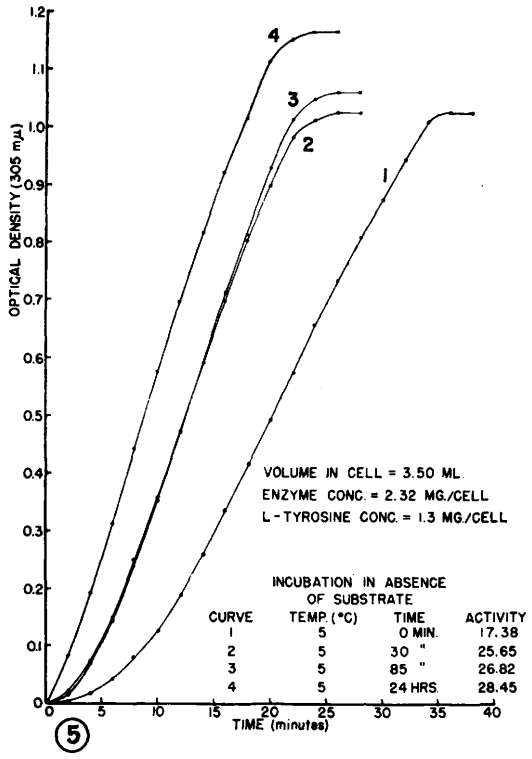
Incubation time (min.)	Exp. 1			Exp. 2		
	Tyrosine utilized (μmoles/ml)		Dopachrome produced (μmoles/ml)	Tyrosine utilized (μmoles/ml)		Dopachrome produced (μmoles/ml)
	Observed	Calculated		Observed	Calculated	
0	0	0	0	0	0	0
5	.0036	.0078	.0078	.0069	.0104	.0104
10	.0285	.0313	.0312	.0345	.0342	.0341
15	.0755	.0560	.0558	.0620	.0621	.0618
20	.0810	.0783	.0780	.0841	.0858	.0855
25	.1060	.1073	.1069			
30				.1284	.1354	.1349

copper(5), and recovered quantitatively by elution from the paper with methanol. The optical density at 512 mμ of these methanol eluates is linearly related to amount of tyrosine initially deposited on the chromatographic paper in the range of 1.3 to 25.0 μg, thus affording a convenient method for estimation of free tyrosine in a mixture. Reaction mixtures were prepared in sets of 6, containing equal amounts of enzyme extract and equal amounts of tyrosine. These were incubated and optical density measurements at 475 mμ, measuring dopachrome concentration, were made at precisely 2 minute intervals. During the experiment, and at time intervals of 5, 10, 15, 20, 25, and 30 minutes, the reaction was arrested in a single cell by mixing its contents with an equal volume of absolute ethanol. At end of experiment, each mixture was subjected to chromatography and the amount of tyrosine remaining determined by the method described above. The amount of tyrosine utilized was then determined by difference.

Fig. 4 clearly demonstrates that the relationship between tyrosine utilized and increase of optical density at 475 mμ is a linear one. The relationship between tyrosine utilized in a reaction mixture and its increase in optical density, as determined by the least squares method, is tyrosine utilized (μmoles/ml) = 0.290 X optical density increase (475 mμ). The linearity of this relationship suggests that there are no other pathways of tyrosine utilization in the reaction mixtures, and that there is an equimolar relationship between amount of tyrosine utilized and amount of dopachrome produced. This last conclusion is more directly demonstrated by calculating the amount of dopachrome produced from the observed increase in optical density and the molecular extinction coefficient value of dopachrome as given by Mason(6). Table I compares such values with both the observed amount of tyrosine utilized and the amount of tyrosine utilized as calculated from the relationship presented in Fig. 4. Since there is no significant difference between amount of tyrosine utilized (observed or calculated) and amount of dopachrome produced, it may be concluded that an equimolar relationship exists between the 2, and that the use of crude extracts and spectrophotometric methods is justified.

Analysis of induction period. The induction period observed in oxidation of tyrosine by purified tyrosinase is generally attributed to the necessity for a catalytic amount of dihydroxyphenol for the reaction to proceed(7). Our crude extracts, however, contain protyrosinase which undergoes enzymatic activation during incubation, even in the absence of substrate(4). The induction period which we observe could be attributable to both an initial absence of required dihydroxyphenol, and to an actual increase in amount of enzyme during the reaction due to protyrosinase activation.

That this is indeed the case is demonstrated by the effects of incubation of enzyme extract at 5°C in the absence of substrate (Fig. 5). During the first 30 minutes of incubation enzyme activity (*i.e.*, slope of linear portion of curve) increases and the induction period is shortened. Other work(4) has demonstrated that most of the protyrosinase undergoes activation during this period. It is therefore



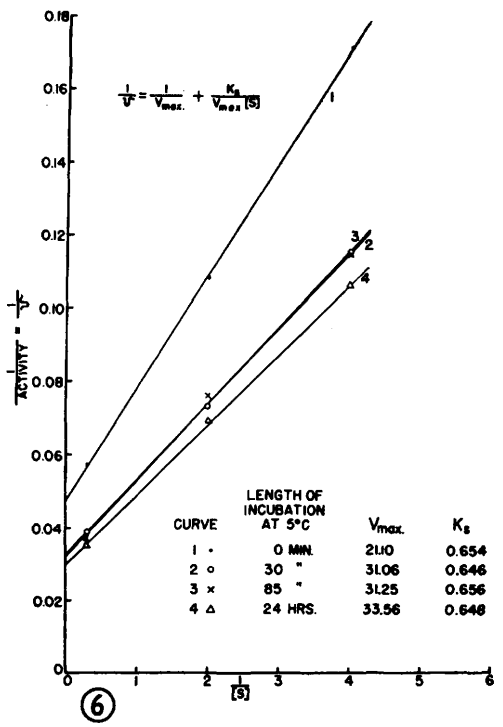


FIG. 5. Effects of incubation of 15300-131*a* extract in absence of substrate.
FIG. 6. Effects of incubation of 15300-131*a* extract in absence of substrate on Lineweaver-Burk kinetics of subsequent tyrosine oxidation.

not surprising to note that further incubation results in no further increase in enzyme activity, but does lead to eventual disappearance of the induction period.

These conclusions are better demonstrated when the data are subjected to Lineweaver-Burk treatment (Fig. 6). V_{max}, which under these conditions measures enzyme concentration, increases during the first 30 minutes of incubation, but undergoes no further change in spite of progressive shortening of the induction period. It is interesting to note that K_s does not change during incubation, thus confirming our previous conclusion(4) that the tyrosinase resulting from protyrosinase activation is the same as that originally present.

It may therefore be concluded that shortening of the induction period during the first 30 minutes of incubation is largely attributable to conversion of protyrosinase to tyrosinase, while the subsequent disappearance of the induction period is probably the result of the slow production of dopa or some other dihydroxyphenol from small amounts of precursors present in the extract. When a fresh extract is tested for tyrosinase activity, both mechanisms will contribute to the observed period of induction.

Summary. 1. Oxidation of tyrosine by crude enzyme extracts of *Neurospora* mycelium, as measured by spectrophotometric determination of dopachrome formation, follows a course similar to that exhibited by purified tyrosinase. 2. The reaction exhibits conventional Michaelis-Menten kinetics with a pH optimum of 7.4. 3. Each mole of tyrosine utilized during the reaction is converted into a mole of dopachrome, thus demonstrating that no enzymatic systems utilizing tyrosine other than tyrosinase are present, and that the use of crude extracts and spectrophotometric methods is justified. 4. The induction period exhibited during the oxidation of tyrosine by the crude extracts results from both the production of catalytic amounts of dihydroxyphenols and conversion of protyrosinase to tyro-

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Antiaccelerator, Coronary Dilator and Certain Other Pharmacologic Actions of New Poly-Methoxyphenyl Derivatives. (23957)

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A specific new pharmacologic activity, the cardiac antiaccelerator effect, was described by Krayer and his associates (for review, *cf.* 1). This activity was found among naturally occurring veratramine and solanum alkaloids (1,2). More recently, a similar effect was described by Margolin and his associates(3) in the case of certain synthetic steroids with alkamine substitutions in position 16. It was conceived that relatively simple polyphenyl molecules with a lipophilic appendage may exhibit similar pharmacologic properties. This paper describes antiaccelerator potency data obtained in the course of screening of a series of such compounds as well as additional pharmacologic and toxicity results obtained with 2 particularly promising compounds of this series.

Methods. The blockade of positive chronotropic effect of epinephrine was tested in rabbit heart Langendorff preparations perfused at 25°C with oxygenated (95% O_2, 5% CO_2) Ringer-Locke by means of an apparatus (4) that permitted measurement of coronary flow. Cardiac rate was measured by means of Palmer drop recorder and amplitude of cardiac beat by means of a light isotonic lever. Epinephrine bitartrate was prepared in Ringer-Locke (5-10 μg/cc) with sodium bisulfite, final concentration 0.1%, acting as antioxidant. Epinephrine was perfused at 1 cc/min. through a side arm provided with a flow meter. The compounds tested were solubilized in weak HCl and injected in 0.1 to 0.2 cc into the perfusion cannula near the heart. The reference compound, veratramine, was solubilized as described by Krayer(5); veratramine doses refer to the base. The positive chronotropic effect of epinephrine amounted in 20 tests to an average of 36 beats/minute and decreased by an average of 5 beats, or 14%, at end of 2 to 4 hours. In actual testing, inhibition of acceleration of 30% or more was considered significant. Anti-accelerator doses effective in 50% of hearts (AED_{50}) were determined from a dose-frequency curve according to the method of Wright(6); 3 to 5 doses were spaced at 0.5 logarithmic intervals, 3 to 5 hearts/dose. Additional measurements of the effect of compounds tested on coronary flow were carried out by the method of Luduena, Miller and Wilt(7). The acute LD_{50} values and their standard error were measured in mice by the method of Miller and Tainter (8). Irritancy was determined in rabbits by means of trypan blue test(9,10). Local anesthetic potency was determined in guinea pigs by the intradermal wheal test(11). The cardiac rate was measured in atropinized dogs under chloralose or barbiturate anesthesia by means of a Palmer Recorder and a pressure transducer(12). The blockade by the com-

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