

A COMPETITIVE INHIBITOR OF TYROSINASE

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Summary

The kinetics of the activation of catechol by tyrosinase prepared from the potato and the mushroom, and of its inhibition by sodium *m*-hydroxybenzoate, have been studied. The enzyme-substrate dissociation constants differed markedly between the two enzyme sources (K_s potato = 5.0mM, K_s mushroom = 0.28mM), as did also the enzyme-inhibitor dissociation constants (K_i potato = 2.5mM, K_i mushroom = 0.6mM). For both enzyme preparations sodium *m*-hydroxybenzoate met the requirements of a competitive inhibitor.

I. INTRODUCTION

Although there has been a considerable amount of work done on the enzyme tyrosinase, it has been mainly concerned with the distribution of the enzyme in plant or animal species (see e.g. Onslow 1931) or, more frequently, with the mechanism of the action of the enzyme on monohydric and dihydric phenols (reviewed in Nelson and Dawson 1944). A number of workers in the field have noticed inhibition of the enzyme by various substances, mainly those reacting with copper, but few have tried to identify the type of inhibition.

A search of the literature has revealed only one paper that examined the kinetics of tyrosinase according to the theory of Michaelis and Menten (1913) and its inhibition by a competitive inhibitor. This is a paper by Hackney (1948) on the tyrosinase of apples, and it is unfortunate that misconceptions of the criteria of competitive inhibition and some apparent mathematical errors should have marred the work; these will be discussed later.

This paper records a study of a competitive inhibitor of tyrosinase on more valid grounds. A preliminary examination of a number of possible aromatic inhibitors led to the selection of *meta*-hydroxybenzoic acid as one which inhibits the enzyme strongly and is apparently free of side effects; resorcinol did not appear to be satisfactory in the latter respect.

II. MATERIALS AND METHODS

Substrate.—Catechol, B.D.H. laboratory reagent "Pyrocatechol" was used.

Inhibitor.—Sodium *m*-hydroxybenzoate was prepared by neutralizing solid *m*-hydroxybenzoic acid (B.D.H. laboratory reagent) with the calculated amount of 10 per cent. NaOH and evaporating and allowing to crystallize. The pH of a solution of this salt was about 7.2.

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Enzyme.—Studies were commenced with a preparation from the common potato, *Solanum tuberosum*. Frozen potatoes were peeled, sliced, and blended in a Waring Blendor with enough 1M phosphate buffer of pH 7.5 to make the final concentration 0.001M. The mix was then filtered through Whatman No. 4 filter paper into *n*-butanol, keeping the whole system as cool as possible. The flocculent precipitate was filtered off and the excess butanol removed. A clear, brownish solution, stable in the refrigerator for several months if protected from air, resulted. No attempt was made to purify or concentrate this solution any further. The activity of the solution, measured according to the method of Miller *et al.* (1944) was about 20-40 units per ml. depending on the batch. For this investigation, several batches were pooled.

Later work was done with a commercial preparation of purified tyrosinase from the mushroom, *Psalliota campestris*, produced by the Syn-Zyme Laboratories of New York. This solution had an original activity of 3700 units per ml., and was diluted for use to about 30 units per ml.

Measurement of activity.—The common methods of measuring tyrosinase activity were all developed to study the concentration or activity of various enzyme preparations, not the effect of varying the concentration of substrate. The actual substrate concentrations used by the various authors were either completely arbitrary or else adjusted to that concentration which would give maximum activity, the "optimum concentration" of Nelson and co-workers. This is in effect the concentration of substrate at which the normal Michaelis curve shows no further appreciable rise. In all cases the methods as published demanded concentrations of substrate above those desirable for kinetic work. As a consequence, several different methods were studied, using low concentrations of substrate.

The methods examined were:

(1) *Manometric Method*.—Warburg manometers and vessels were used, with a reaction mixture of enzyme, substrate, and 0.07M phosphate buffer of pH 7.4 to a total volume of 3.0 ml. The temperature was maintained at 25°C. In some experiments 0.1 per cent. gelatin (Nelson and Dawson 1944) or *o*-phenylene diamine in equimolar concentrations to substrate (Richter 1934) was added.

(2) *Colorimetric Method*.—Developed in the course of some unpublished work by J. E. Humpoletz to determine tyrosinase concentration. The reaction mixture was 1.0 ml. of enzyme plus substrate and inhibitor and 0.001M phosphate buffer of pH 7.5 up to 10.0 ml. This was immediately examined in an E.E.L. photoelectric colorimeter using a blue filter (No. 303) and the optical density recorded every 15 sec. for 3-5 min. This was done at room temperature, about 22°C. When the optical density was plotted against time, a straight line was obtained, at least for the period ½-2½ min. The velocity of the reaction was recorded as the increase in optical density multiplied by 100 per min.

(3) *Chronometric Method*.—This was used by Miller *et al.* (1944). The reaction mixture was 2.0 ml. of enzyme plus substrate, inhibitor, and ascorbic acid, made up to 100 ml. with 0.001M phosphate buffer of pH 7.5. The action took place at room temperature, about 22°C. The velocity of the reaction was measured in ml. of ascorbic acid solution oxidized per sec.

III. RESULTS

(a) *Examination of Methods of Measuring Tyrosinase Activity*

As with previous workers (see Nelson and Dawson 1944, p. 117), difficulty experienced in maintaining a reasonably constant rate of oxygen uptake in the manometric method prevented accurate estimation of the initial reaction rate. Addition of gelatin or of *o*-phenylene diamine lessened but did not eliminate this difficulty; in the latter case some inhibition was also noted. Under no conditions could strictly linear relationship between enzyme concentration and initial velocity be obtained, even at extreme dilutions of enzyme and moderate substrate concentration (1mM catechol); this is undesirable for kinetic studies. Further, this technique measures the uptake of oxygen in at least two steps (Nelson and Dawson 1944), so that the rate measured is the combination of several rates, of which not all may be under enzymic control. Because of these difficulties, the technique was abandoned.

Preliminary experiments using the colorimetric technique and *p*-cresol, catechol, and pyrogallol as substrates at concentrations of 0.02-0.05mM and sodium *m*-hydroxybenzoate, phloroglucinol, and KCN as inhibitors and the potato enzyme gave apparently satisfactory figures in accordance with theory, showing sodium *m*-hydroxybenzoate and phloroglucinol as competitive inhibitors and KCN as a non-competitive inhibitor. Apparent Michaelis constants K_s^* for each substrate were of the order 0.1-0.5mM and inhibitor dissociation constants K_i for the competitive inhibitors were of the order 0.3-2.0mM.

However, when the substrate concentrations were increased serious anomalies and side effects were noted. Using catechol as substrate, the graph of velocity against substrate concentration did not become progressively flatter as the substrate concentration was raised above 1.0mM, as would be expected for a typical Michaelis curve, but it approximated to a straight line with a marked slope (Fig. 1). At the same time, some qualitative differences in the colour developed were noticed.

This colour is due to the combined effect of several compounds: the primary and, particularly, the secondary oxidation products of the substrate and their compounds with other substances in the medium, such as amino and imino acids (Jackson and Kendal 1949; Trautner and Roberts 1950). Differences in the reaction mixture, such as those between the potato enzyme with

* The following symbols are used throughout: E = enzyme concentration; S = substrate concentration; I = inhibitor concentration; v = velocity of the reaction; V = velocity of the reaction when the enzyme is saturated with substrate; K_s = Michaelis constant, the dissociation constant of the enzyme-substrate complex; K_i = dissociation constant of the enzyme-inhibitor complex.

its accompanying impurities and the relatively pure mushroom enzyme, might be expected to (and in fact were found to) lead to differences in the colour developed. But it was also noticed that, concurrent with the change from apparent conformity with Michaelis' theory to obvious non-conformity, increasing substrate concentration led to a qualitative change in colour. Between substrate concentrations of 1 and 5mM (with catechol) the colour changed progressively from red-brown to bright yellow; above 5mM, the colour remained qualitatively the same, though its rate of development increased approximately linearly. It should also be noted that, as with the manometric method, this technique measures components of several different reaction rates simultaneously. These considerations necessitated the abandonment of the method despite its very considerable saving of time compared with other methods of measuring tyrosinase activity.

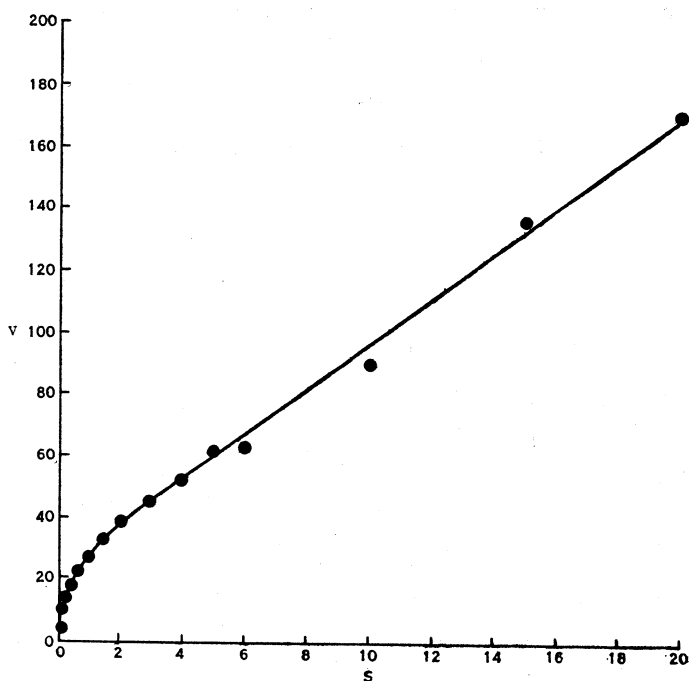


Fig. 1.—Relationship between enzyme action and substrate concentration for the potato preparation as measured by the colorimetric technique. Reaction velocity v measured in increase in optical density units per minute plotted against substrate concentrations expressed as millimolarity of catechol. The line was fitted by eye.

The chronometric technique has many advantages: it measures the rate of the primary oxidation of catechol to *o*-quinone, and gives a close approximation to the initial rate. However, it will measure only fairly rapid reaction rates within a limited range. The method would be more suitable to kinetic

studies if a more sensitive method for the detection of *o*-benzoquinone were available. It was, however, obviously the method of choice for the experiments that follow.

It is of interest to note that the colour developed in the reaction mixture after the oxidation of the ascorbic acid had taken place was qualitatively different from that developed in a similar mixture without the ascorbic acid.

(b) Kinetic Studies

Results for the potato enzyme are recorded graphically in Figure 2, as velocity against substrate concentration (Fig. 2A) and according to the first modification of Lineweaver and Burk (1934), as $1/v$ against $1/S$ (Fig. 2B). It proved difficult to get reproducible results using this enzyme source and only the most reliable results are plotted. More satisfactory results were obtained for the mushroom enzyme, and the data for a typical experiment are plotted in similar fashion in Figure 3.

IV. DISCUSSION

Theoretically, it is to be expected with competitive inhibition that increasing the concentration of substrate will decrease the proportionate inhibition by a given concentration of inhibitor, so that a concentration of substrate should be reached at which the velocity approximates to the maximum velocity obtained in the inhibitor's absence. Experimental limitations, as in the present work, often prevent the point being tested, but the treatment of Lineweaver and Burk (1934) can be readily applied. According to their first modification, when $1/v$ is plotted against $1/S$, the lines with and without inhibitor should have the same intercept, namely $1/V$, when the inhibition is competitive. Non-competitive inhibitors will give lines with intercepts and slopes increased in the same proportion, the intercept becoming $1/V (1 + I/K_i)$, and the slope having the same formula as with a competitive inhibitor, namely $K_s/V (1 + I/K_i)$, to be compared with the slope in the absence of inhibitor, K_s/V .

Working with resorcinol as inhibitor and an enzyme preparation from apples, the general trend of the figures obtained by Hackney (1948) for percentage inhibition on increasing substrate concentration would indicate some element of competition, but her Figure 10, plotted according to the treatment of Lineweaver and Burk, does not demonstrate simple competitive inhibition, in that the intercept does not remain unchanged when inhibitor is present. She calculates K_i as 0.000002 (presumably molar). It has been difficult to check this calculation as the different sets of data yield different values for the Michaelis constant (not noted by Hackney), and, as noted above, the theoretical maximum velocity is different in the presence and absence of inhibitor. However, using average values, the present author re-calculates K_i as of the order 0.1M, a value that invalidates Hackney's discussion of some observed anomalies in the competitive behaviour of resorcinol.

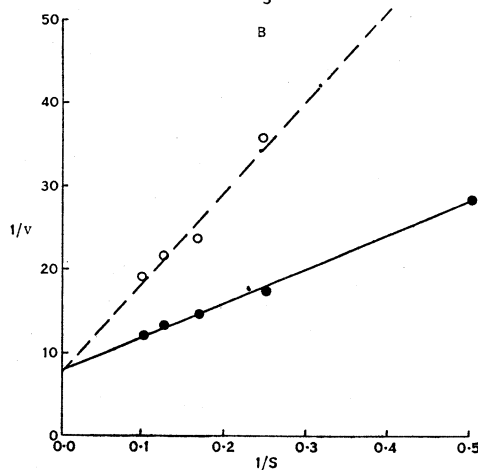
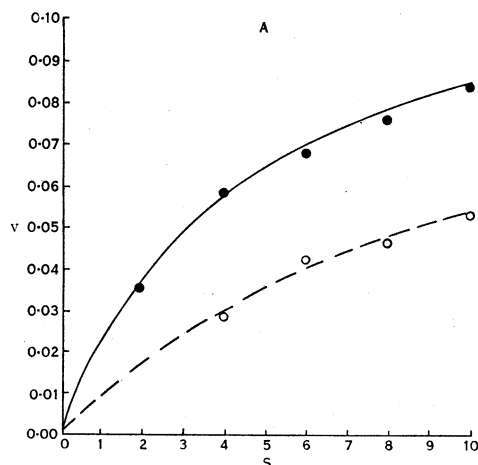


Fig. 2

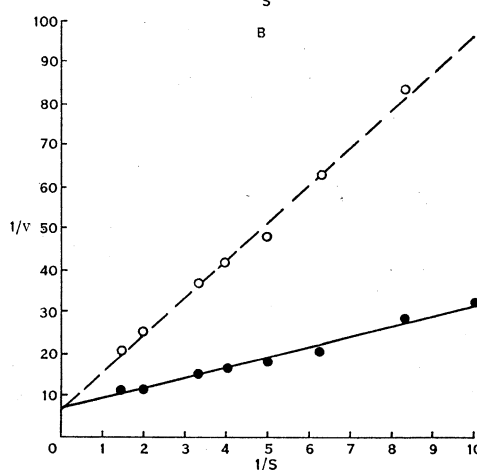
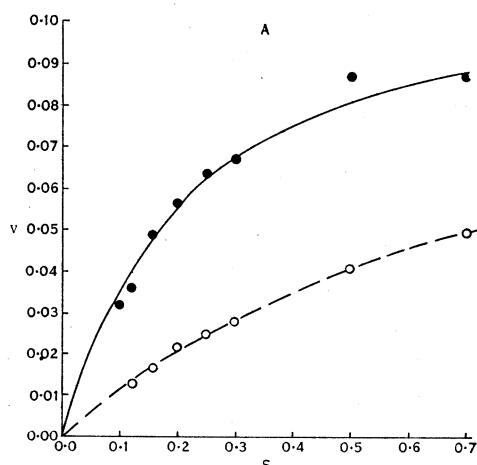


Fig. 3

Fig. 2.—Kinetics of the action of the potato preparation in the presence and absence of 4.0mM sodium *m*-hydroxybenzoate. The reaction velocities v were measured by the chronometric method and expressed in ml. of ascorbic acid solution oxidized per sec. The substrate was catechol and its concentration S expressed as millimolarity. Full lines, uninhibited enzyme; broken lines, inhibited enzyme.

A: Reaction velocity plotted against substrate concentration. Lines drawn through points calculated from Figure 2B.

B: Lineweaver and Burk graphs, $1/v$ plotted against $1/S$. Lines fitted by the least squares method.

Fig. 3.—Kinetics of the action of the mushroom preparation in the presence and absence of 2.0mM sodium *m*-hydroxybenzoate. The reaction velocities v were measured by the chronometric method and expressed in ml. of ascorbic acid solution oxidized per sec. The substrate was catechol and its concentration S expressed as millimolarity. Full lines, uninhibited enzyme; broken lines, inhibited enzyme.

A: Reaction velocity plotted against substrate concentration. Lines drawn through points calculated from Figure 3B.

B: Lineweaver and Burk graphs, $1/v$ plotted against $1/S$. Lines fitted by the least squares method.

It may be noted that Hackney's expectation that, with a non-competitive inhibitor, percentage inhibition would show a linear relationship with inhibitor concentration is in error. It can be readily shown that the curves for competitive and non-competitive inhibitors are both rectangular hyperbolae. Indeed, Hackney's own figures for a non-competitive inhibitor, KCN, as shown in her Table 4, give a curve and not a straight line if plotted in this fashion.

In the present work, it will be seen that sodium *m*-hydroxybenzoate meets the requirements of a competitive inhibitor of tyrosinase performing the activation of catechol, with the constants:

	Potato Enzyme	Mushroom Enzyme
Michaelis constant K_s	about 5mM	0.28mM
K_i	about 2.5mM	0.6mM

The different dissociation constants found for the enzyme prepared from potato and that from mushroom would indicate that either the enzymes had different properties or possibly the preparation from the potato contained a competitive inhibitor of tyrosinase that was lacking in the relatively pure mushroom preparation.

V. ACKNOWLEDGMENTS

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VI. REFERENCES

- HACKNEY, F. M. W. (1948).—*Proc. Linn. Soc. N.S.W.* 73: 439.
 JACKSON, H., and KENDAL, L. P. (1949).—*Biochem. J.* 44: 477.
 LINEWEAVER, H., and BURK, D. (1934).—*J. Amer. Chem. Soc.* 56: 658.
 MICHAELIS, L., and MENTEN, M. L. (1913).—*Biochem. Z.* 49: 333.
 MILLER, W. H., MALLETT, M. F., ROTH, L. J., and DAWSON, C. R. (1944).—*J. Amer. Chem. Soc.* 66: 514.
 NELSON, J. M., and DAWSON, C. R. (1944).—*Adv. Enzymol.* 4: 100.
 ONSLOW, M. W. (1931).—“The Principles of Plant Biochemistry.” (Cambridge University Press.)
 RICHTER, D. (1934).—*Biochem. J.* 28: 901.
 TRAUTNER, E. M., and ROBERTS, E. A. H. (1950).—*Aust. J. Sci. Res. B* 3: 356.