Inhibition of the Tyrosinase Oxidation of One Substrate by Another

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ABSTRACT The catalytic oxidation of catechol by crude preparations of mushroom tyrosinase was studied by a method yielding data on initial reaction velocities. Graphical analysis of the results suggests that an excess of catechol inhibits its own oxidation by a competitive process, thus accounting for the observed optimum in the substrate concentration. However, added phenol, though itself a substrate, inhibits the enzymatic oxidation of catechol by a process that is neither competitive nor non-competitive, but a mixture of the two types. Mechanisms of this inhibition of the enzyme by a second substrate are discussed in exploring the problem of substrate-substrate inhibition.

Tyrosinase catalyzes the oxidation of monohydric phenols, as well as *o*-dihydric phenols. Yet phenol inhibits the action of tyrosinase on catechol (1). The nature of this inhibition of one substrate by another of a different class is, therefore, of interest as one facet of the general problem of enzyme function.

References to the many reports on inhibitors of tyrosinase action are reviewed by Lerner (2). Tyrosinase, a copper-bearing phenolase, is inhibited by compounds which complex with copper, by analogs which competitively inhibit its action, and by metals which compete with the copper. Few papers have dealt with the mechanism of inhibition using Michaelis-Menton kinetics.

Kendal (3), using a manometric method, demonstrated that the addition of phenol competitively inhibits the oxidation of catechol. However, in the presence of catechol, phenol itself is rapidly converted to catechol by the action of tyrosinase and might complicate the interpretation of data from the relatively slow manometric assay he used. Later, Warner (4) showed that *m*-hydroxybenzoic acid acts as a competitive inhibitor in the tyrosinase-catalyzed oxidation of catechol. Hackney (5) studied the inhibitory effect of resorcinol on tyrosinase, manometrically. However, her interpretation was questioned by Warner.

Recently Schneider and Schmidt (6) published data concerning the competitive inhibition by resorcinol, orcinol, and phloroglucinol of the tyrosinase-catalyzed oxidation of *p*-cresol. These compounds are analogs, but not sub-

strates for tyrosinase. In addition, Heymann *et al.* (7) demonstrated that the enzymatic oxidation of catechol is inhibited by still other non-substrates, 4-chlororesorcinol, and *N*-phenylthiourea. The mechanism of these inhibitions is neither competitive nor non-competitive.

With the hope of contributing to understanding of the control of biological processes, the present exploratory study examines the inhibitory effect of phenol on the oxidation of catechol by crude preparations of tyrosinase, using a chronometric method for enzyme assay. A mechanism of inhibition in this system is suggested from Michaelis-Menten kinetics and interpretations are presented.

EXPERIMENTAL METHODS

Tyrosinase Preparation

One pound batches of the common mushroom, *Psalliota campestris*, were ground in a Waring blendor for 10 seconds with 500 ml of acetone at room temperature. This mixture was immediately transferred to 2 liters of acetone chilled with dry ice. After filtering through a Buchner funnel, the excess acetone was removed by fastening a thin rubber sheet securely over the top of the funnel. The suction of the aspirator combined with the atmospheric pressure squeezed the pulp dry. This dry pulp was frozen with dry ice and stored for 2 hours. The frozen cake was suspended in 600 ml of cold water and allowed to stand overnight in the refrigerator. The resulting crude extract was collected by filtering through a heavy cloth and stored cold for use.

Activity Assay

The activity assay used in this work was the chronometric method developed by Miller and Dawson (8) and later improved by Miller *et al.* (9). After determining the optimum concentration of catechol, the amount of ascorbic acid was varied to permit a plot of $1/Q$ (moles^{-1} of quinone produced) *versus* $1/t$ (seconds^{-1}). In all preliminary experiments this plot gave straight lines as previously demonstrated (9). Hence a single ascorbic acid concentration (3.00 mg/100 ml) sufficed to permit calculation of initial velocities of reaction. The endpoint determination was repeated at least four times for each experimental variation.

The initial velocity at the optimum concentration of catechol was recalculated arbitrarily to 0.500 μmoles/sec. in order to compensate for the varying strengths of the different enzyme preparations.[1] The factor thus obtained for adjusting the optimum rate to 0.500 μmole/sec. was then used to compute all the other initial velocities to a comparable basis.

[1] The actual reaction rates at the optimum catechol concentrations for the preparations used in these studies range from 0.393 to 0.511 μmole/sec. Each separate experiment is based on a single enzyme preparation and is, therefore, self-consistent.

RESULTS AND DICUSSION

Table I presents the results of a typical study of the interaction of catechol and phenol during the oxidation of the former by tyrosinase. Inhibition is indicated by rates below 0.500 μ mole/sec. At least partial reversal of the inhibition by phenol is indicated in this table by the tendency for the maximum observed initial velocity to shift to a higher concentration of catechol than characteristic for the systems without phenol.

Data from a similar experiment are presented graphically in Fig. 1. Values of V, K_{EI}, and K_{ES}[2] were calculated from the slopes and intercepts of this type of Lineweaver-Burk (10) plot. Since the lines do not intersect on either the

TABLE I

EFFECT OF CONCENTRATION CHANGES ON INITIAL REACTION VELOCITIES*

Catechol, mg.	Phenol, mg											
	0	25	50	75	100	150	200	300	400	500	600	800
25	0.376	0.364	0.353	0.313	0.270		0.217					
50	0.426	0.408	0.396		0.348	0.326		0.257				
100	0.4835				0.412		0.362	0.331	0.300	0.278		
150	0.500				0.434		0.3695	0.339	0.324	0.312		
200	0.476						0.425	0.391	0.359	0.343	0.321	
300	0.406					0.350		0.322	450‡ / 0.297		0.286	0.261
400	0.3875						0.350		0.311		0.294	0.272

* The initial velocity is recalculated to 0.500 μmoles/second at the optimum catechol level (150 mg/100 ml) and without added phenol (Experiment I).
‡ This point was based on 450 mg of phenol.

$1/v$ or the $1/[S]$ axis, the inhibitory effect of the added phenol cannot be designated as either competitive or non-competitive. In the present experiments, it appeared to be either an intermediate situation or a combination of both.

Comparisons of values for K_{ES}, K_{EI}, and maximum velocity for each of three separate experiments using different enzyme preparations appear in Table

[2] V, K_{EI}, K_{ES}, $[S]$, and v are, respectively, the maximum velocity, dissociation constants for enzyme-inhibitor and enzyme-substrate complexes, molar substrate concentration, and the observed, initial reaction velocity according to

$$\frac{1}{v} = \left(\frac{K_{ES}}{V}\right)\left(\frac{1}{[S]}\right) + \frac{1}{V}$$

$$\frac{1}{v} = \left(\frac{K_{ES}}{V}\right)\left(1 + \frac{[I]}{K_{EI}}\right)\left(\frac{1}{[S]}\right) + \frac{1}{V}$$

II. K_{EI} was calculated on the basis of competitive inhibition although it appears graphically that the inhibition is neither competitive nor non-competitive. The variations in observed values of K_{EI} between individual experiments are probably due to differences of aging and impurities in the tyrosinase preparations. Differences in the values of K_{ES} are probably within experimental error.

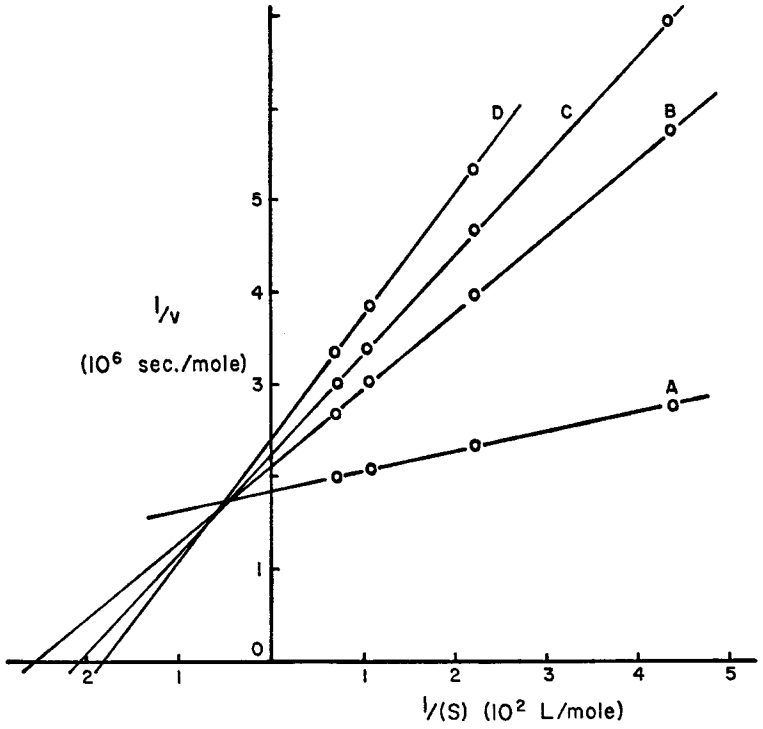

FIGURE 1. The mechanism of inhibition of catechol oxidation. The concentration of ascorbic acid oxidized indirectly by tyrosinase in the presence of catechol varied from 2.27 to 18.16 mM (Experiment III). Line A, no phenol; line B, 21.25 mM phenol; line C, 31.88 mM phenol; line D, 42.5 mM phenol. The points as drawn are larger than the average deviations in the data.

To determine by graphic means a more exact value of K_{ES}, the substrate concentration [S] is plotted (11) *versus* [S]/v (Fig. 2) yielding K_{ES} = 1.0 mM. A linear plot is obtained for concentrations from low levels through the optimum. For greater concentrations of catechol, a curve is obtained which can be made linear (12) upon plotting v/[S] *versus* v[S] as in Fig. 3. From this latter plot the K'_{EI}, the complex number for the inactive *SES* complex, was calculated to be 0.65 M, a 500-fold increase over K_{ES}. This increase is to be expected from the [S]/v *versus* [S] curves (Fig. 2) where it can be seen that the inhibition by an excess of catechol is slight. This discontinuitv, or curvature, in Fig 2 suggests

that there should be a change in K_{ES} or K'_{EI} in switching from one segment of the curve to the other.

As evidenced by the preceding data, the mechanism of the inhibition of catechol oxidation by phenol is neither competitive, as Kendal had previously suggested, nor non-competitive. Dixon and Webb (13) outlined the possibili-

TABLE II

LINEWEAVER-BURK CONSTANTS ASSUMING COMPETITIVE INHIBITION IN THE OXIDATION OF CATECHOL

Constant	Phenol	Experiment		
		I	II	III
	mM			
V, *μmole/sec.*	0	0.53	0.53	0.54
K_{ES}, *mM*	0	0.95	0.96	1.1
K_{EI}, *mM*	10.6	9.7	6.1	
	21.3	9.8	6.9	7.1
	31.9	10.0	7.1	7.7
	42.5	9.2	8.2	7.8

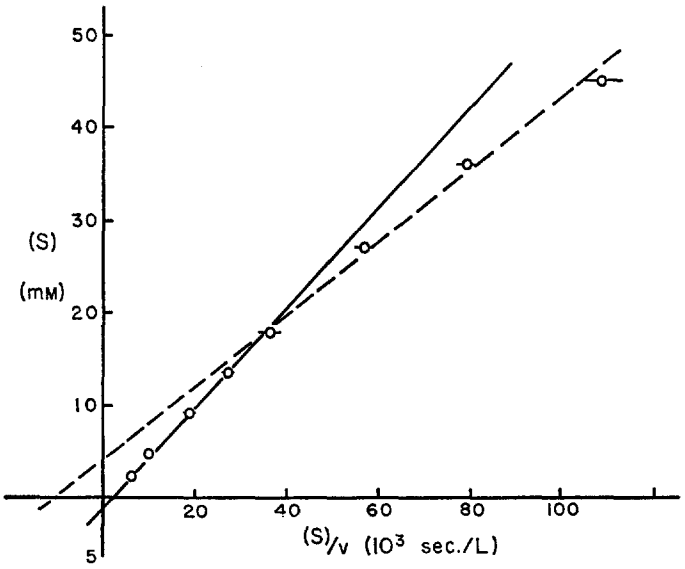

FIGURE 2. Inhibition of the oxidation of catechol by an excess as shown by a plot based on Experiment II. No phenol was added for these points. The horizontal lines indicate the average deviations in experimental values. The solid line represents the concentration range up to the optimum, and the dashed line, the range beyond the optimum which is about 14 mM.

ties for the mechanism of such systems. The interaction may be partially competitive, partially non-competitive, or a mixture of types.

Plots of $1/v$ *versus* $1/[S]$ show variations in V as well as in K_{ES}. Such variation eliminates the possibility of either partially non-competitive or partially competitive inhibition, since neither of these can be distinguished from the pure types by Lineweaver-Burk plotting. Therefore, the occurrence of a mixture of the two types of inhibition is a distinct possibility. In a mixture of types, both V and K_{ES} should change with an increase in reaction velocity.

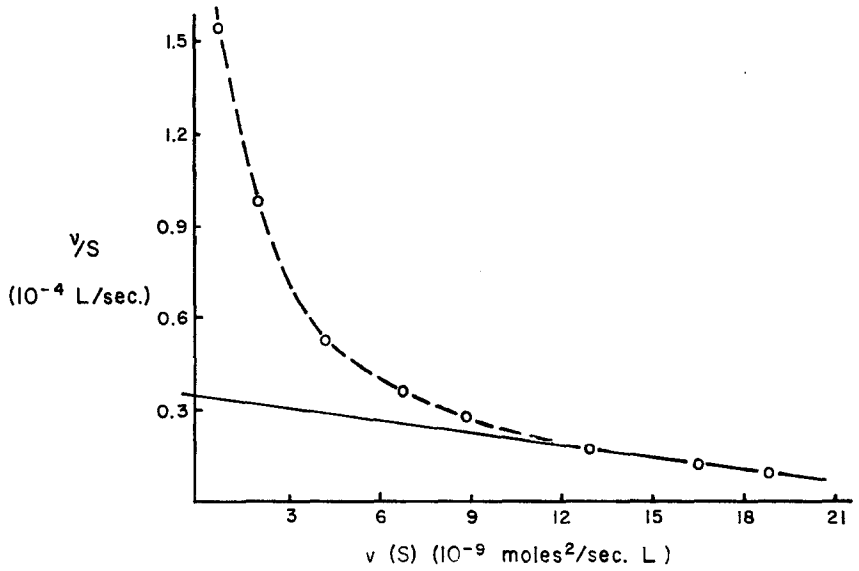

FIGURE 3. Competitive inhibition by excess substrate in the oxidation of catechol. No phenol was added to these systems. The points as drawn are larger than the average deviations in the data (Experiment II).

Friedenwald and Maengwyn-Davies (14) treated the case of a mixture of partially competitive and non-competitive inhibitions. They made the assumption that the inhibitor alters the affinity of the enzyme for the substrate. Moreover, this effect on affinity must also occur in the breakdown of the enzyme-substrate complex as well as during the formation. The above treatment accounts for the present graphical results and values of the dissociation constants plotted and calculated according to Lineweaver and Burk (10).

Dressler and Dawson (15), working with Cu^{64}, suggested that the enzymatic sites for phenol and catechol are different. Such a difference could account for the lack of simple, competitive inhibition by the phenol. Nevertheless, close proximity of the sites for the two classes of substrates might explain the partially competitive, partially non-competitive interference observed.

On the other hand, the possible presence of naturally occurring protein inhibitors (16) in these crude preparations might influence the kinetics, and the problem should be reexamined with a series of highly purified preparations. Existence of different sites for the phenol and catechol activities could lead to the results obtained if both types of sites are affected by phenol.

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