Physicochemical and Kinetic Properties of Mushroom Tyrosinase*

HARRY W. DUCKWORTH† AND JOSEPH E. COLEMAN

From the Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06510

SUMMARY

Tyrosinase (o-diphenol: O₂ oxidoreductase, EC 1.10.3.1) has been purified from a commercial lyophilized powder prepared from edible mushrooms. Specific activities toward DL-dihydroxyphenylalanine and catechol, homogeneity, molecular weight, absorption spectrum, amino acid composition, and copper content of the preparation are comparable to other purified samples of tyrosinase prepared from fresh mushrooms. Circular dichroism of the enzyme reveals an unusually shaped envelope of negative ellipticity bands in the deep ultraviolet (106 to 250 mμ), which is difficult to explain by a combination of the usual peptide conformational forms. Small positive ellipticity bands ([θ] per 32,000 mol wt ≈ 30,000) are associated with the near ultraviolet absorption of the aromatic amino acid side chains.

Kinetic constants for the oxidation of a homologous series of catechol substrates substituted in position 4 by groups —H, —SCN, —COCH₃, —CHO, —CN, and —NO₂ have been determined. As the electron-withdrawing ability of the substituent increases, Kₘ and kₐₑₜ decrease in the order H > SCN > COCH₃ > CHO > CN > NO₂. Except for the kₐₑₜ values for the two most slowly oxidized substrates, both Kₘ and kₐₑₜ conform to Hammett relationships when the substituent parameter ρ is used. The ρ values are −1.01 ± 0.09 for Kₘ and −2.49 ± 0.11 for kₐₑₜ. The oxidation of pyrocatechol, 4-COCH₃, and 4-CHO-catechol give a satisfactory isokinetic relationship between ΔH° and ΔS°. These findings suggest that these substrates are oxidized by the same basic mechanism.

Studies of the dependence of catechol oxidation on the concentration of oxygen show that Kₘ for oxygen varies with the nature of the catechol substrate. With the exception of the pyrocatechol complex, the enzyme-substrate complexes for this series of catechols are saturated in air (20.95% O₂). The oxygen kinetics suggest a sequential mechanism for the binding of catechol and oxygen. Detailed studies of the pH dependence of Kₘ and kₐₑₜ show the presence of a group in the enzyme whose pKₐ changes upon the binding of a catechol molecule.

Inhibition of tyrosinase by benzoic acid and cyanide show that the former is competitive with catechol and noncompetitive with oxygen, while the latter is competitive with oxygen and noncompetitive with catechol. These results are taken to indicate two distinct substrate binding sites on the enzyme, one with a high affinity for aromatic compounds including phenolic substrates, the other for metal-binding agents and oxygen. The latter site presumably involves enzyme copper. Binding studies with 14C-benzoic acid suggest that there are two binding sites for benzoic acid per mole of tetramer (mol wt = 128,000). The equilibrium constant for this binding is the same as the Kᵣ measured for competitive inhibition of catechol oxidation by benzoic acid. Catechols are activators of the phenolase reaction of tyrosinase, and tyrosinase is shown to be an inhibitor of this activation of its own oxidation in a manner suggesting that there is a third binding site on the enzyme for an “activator” catechol or phenol.

Enzymes which use molecular oxygen to oxidize phenols and polyphenols have been prepared from various sources (1). Certain of these enzymes contain copper as an integral part of their structure, and one type of copper oxidase which has received a great deal of study is that referred to as tyrosinase, polyphenol oxidase, and “phenolase complex” (2, 3). Enzymes of this type, which have been described in some detail from mushrooms (4–7), potato tubers (8, 9), Neurospora crassa (10), broad bean (11), and mammalian melanoma tumors (12), are able to catalyze two ostensibly different reactions: the ortho-hydroxylation of phenols to give catechols, and the dehydrogenation of catechols to quinones. These latter products are unstable in aqueous solution, and break down over a period of minutes to hours by a combination of nucleophilic reactions and further oxygen-mediated oxidations, not all of which are understood (1).

Mason, Fowlks, and Peterson (13) have shown that in the hydroxylase process, the source of the oxygen of the new hydroxyl group is molecular oxygen, a fact which qualifies the enzyme as a mixed function oxidase. In the case of catechol oxidation, investigations by electron spin resonance techniques indicate that the electrons are transferred not one at a time, but in pairs (14). It is known that 1 molecule of oxygen is able to oxidize 2 molecules of catechol (1). A large amount of information has accumulated regarding the variety of substrates and inhibitors which interact with the enzyme (3).
Information has been slow to accumulate which will help determine the mechanisms by which tyrosinase catalyzes its specific reactions, in part because of the difficulty of preparing the pure enzyme reproducibly and because of the general instability of the enzyme. In this paper we present a simple, rapid method for preparing moderate amounts of pure tyrosinase from mushrooms, taking advantage of a preparation that is available commercially. In addition we also report quantitative studies of the action of tyrosinase on a select number of simple catechol derivatives. The Michaelis-Menten parameters associated with the oxidation of these substances by tyrosinase prove to be very sensitive to the nature of the substituents present on the aromatic rings. Studies of the dependence of these kinetic parameters on oxygen concentration, pH, and the presence of aromatic and metal-binding inhibitors suggest possible relationships between substrate, activator, and oxygen binding sites on the enzyme.

**MATERIALS AND METHODS**

**Reagents**

All chemicals were reagent grade. Water which had been distilled and then passed through a mixed bed ion exchange column to remove metal ions was used routinely for preparation of solutions. Guanidine hydrochloride was prepared from the carbonate according to the method of Kawahara, Kirschner, and Tanford (15). Benzoic acid-carboxyl-14C was obtained from New England Nuclear. Pyrocatechol (Fisher), Dl- and L-dihydroxyphenylalanine (Sigma), and L-tyrosine (Mann) were used without further purification. The other catechol substrates were obtained as follows.

- 4-Acetylacetone (3,4-Dihydroxyacetophenone)—This substrate was prepared according to the method of Rosennund and Lofert (16) and finally recrystallized from xylene, giving a dry product, pale yellow needles, m.p. 119-120° (in the literature, 116° (16)).
- 4-Formylacetone (Protocatechualdehyde)—This was Aldrich technical grade, twice recrystallized from water, m.p. 153-153.5° (in the literature, 153-154° (17)).
- 4-Thiocyanatoacetone—4-Thiocyanatoacetone was prepared according to the method of Macbeth (18), pale yellow plates from water, m.p. 141.5-142.5° (in the literature, 142° (18)).
- Cyanacetone—4-Cyanacetone was prepared according to the method of Hoch and Zarzecki (19), pale tan needles from xylene, m.p. 153-153.5° (in the literature, 156° (19)).
- Nitrocatechol—This substrate was prepared according to the method of Slociof (20), yellow needles from ethanol, m.p. 174-175° (in the literature, 178° (20)).

**Assay Methods**

Spectrophotometric measurements were made on a Cary model 15 double beam instrument with 0.1 slide wire where necessary. Routine activity measurements were made using DL-dopa as substrate according to the method of Fling, Horowitz, and Heinemann (10), except that the reaction mixture was reduced to 2.50 ml of 0.1 M sodium phosphate, pH 7.0, containing 2 mg of DL-dopa. The Δε for the reaction at 475 μM is 3600 (21).

Oxidation of tyrosine was followed at 280 μM, as suggested in the Worthington Biochemical manual; changes in optical density at this wave length are largely due to the tyrosine-dopa conversion, with a slight contribution from the further oxidations of dopa. The Δε for the tyrosine-dopa conversion is 1440 M⁻¹ cm⁻¹.

- α-Benzoquinone, the immediate product of the enzymatic oxidation of pyrocatechol, is reasonably stable in aqueous solutions only at pH 4 or lower. For rate measurements of this oxidation, therefore, the assay method of Sussman (22) was used, in which 0.02 M potassium ferrocyanide is included in the reaction mixture, and its oxidation (Δε per mole per electron = 1020, or per catechol = 2040, at 420 μM) by generated α-benzoquinone is followed. Spectrophotometric measurement of the oxidation of the simple catechol derivatives posed special problems. Pyrocatechol itself is well known to inactivate tyrosinase rapidly as its oxidation proceeds; this makes estimation of the initial rate difficult. We have confirmed that the decline in rate of oxidation of catechol with time, interpreted as irreversible inactivation of the enzyme, accurately follows a first order process during the first 80% of the reaction, and therefore initial rates in these studies were determined by extrapolating a plot of the logarithms of the slopes of the oxidation progress curves versus time to zero time. Ferrocyanide does not react quantitatively with the quinone products of oxidation of the other catechols studied. Therefore direct spectrophotometric assays were developed for the other catechols by determining the spectra of the product quinones (see "Results"). Rates of oxidation of the substituted catechols were always zero order over the extent of reaction studied, and semilog extrapolations were not needed to obtain initial velocities in these cases.

Reaction mixtures were equilibrated with levels of oxygen different in concentration from air by bubbling the appropriate gas phase through the reaction mixture in a Thunberg cuvette (Hillma Cells, Inc., Jamaica, New York) at a rate of 100 to 150 cc per min for 10 min. This equilibration period was enough, when pure nitrogen was used, to reduce the rate of oxidation of catechol by tyrosinase to zero. Gas mixtures were oxygen-nitrogen in known proportions, prepared by The Matheson Company (East Rutherford, New Jersey), or made from these by the use of a Matheson proportionating flow meter. During the equilibration period the enzyme was contained in the side arm with the other reaction components in the cuvette; the reaction was begun by tipping in the enzyme. A special Plexiglas extension, painted black, was designed to enlarge the Cary model 15 sample chamber to allow use of the Thunberg cuvettes in that instrument.

**Assay Buffers**

Sodium phosphate, 0.1 M, pH 7.0, was used for all kinetic measurements except those in which pH was varied. Many buffer anions, which are useful in the pH range 4 to 7, are powerful inhibitors of tyrosinase, particularly below pH 5.5. For the pH-dependent measurements we used a buffer system comprised of 0.025 M succinic acid and 0.025 M tetrasodium pyrophosphate. The pH was adjusted with standard 4 M NaOH or HNO₃, and the calculated amount of 1 M NaNO₂ was added to bring the ionic strength up to 0.10. Succinate, pyrophosphate, and nitrate are among the very few anions reported to be noninhibitory at the concentrations needed for the buffer system (23). Kinetic results obtained with this system at pH 7.0 were quantitatively the same as those obtained in phosphate buffer.
**Equilibrium Dialysis**

Benzoic acid-binding experiments were performed in succinate-pyrophosphate-nitrate buffer at pH 5.0. Aliquots, 0.6 ml, of enzyme of a concentration near 0.32 mg per ml (~10 μM) in monomer contained in a small dialysis bag were placed in 80 ml of buffer containing the labeled benzoic acid. Experiments were equilibrated for 48 hours. Aliquots, 0.40 ml, were then withdrawn by syringe from the dialysis bags and dialysates and injected into scintillation vials containing 15 ml of dioxane scintillation mixture (100 g of naphthalene, 5 g of 2,5-diphenyloxazole (PPO), and 0.125 g of 1,4-bis-[2-(phenyloxazolyl)]-benzene (POPOP) made up to 500 ml with 1,4-dioxane). They were counted in a Packard Tri-Carb liquid scintillation spectrometer.

**Amino Acid Analysis**

A total of six preparations of mushroom tyrosinase was analyzed. Pure protein, 0.1 to 0.3 mg, was hydrolyzed 22 or 72 hours at 110°C in 2 ml of constant boiling HCl, in evacuated, sealed tubes. The hydrolysates were analyzed on the Beckman model 120B and 116 analyzers by the standard methods. Half-cystine was determined by the method of Moore (24), and tryptophan by the method of Edelhoch (25).

**Copper Analysis**

Copper was measured in intact protein samples by atomic absorption analysis at 3247 Å with a Jarrell-Ash spectrometer with attached recorder. Sample volumes of less than 100 μl could be used, in a concentration range of 0.2 to 4 ppm, with the “spike-height” method of absorbance determination described elsewhere (26).

**Measurement of Protein Concentration**

Precise determinations of protein were made by hydrolyzing a known aliquot of a solution for 22 hours in 6 N HCl in an evacuated, sealed tube, and determining the content of lysine, histidine, and arginine on the short column of the amino acid analyzer. Routine measurements of protein concentration made use of the extinction coefficient, $E_{280}^{\text{cm}} = 24.9 \pm 0.3$, at 281 nm, which was based on primary estimates of protein by the amino acid analysis method.

**Ammonium Sulfate**

A total of six preparations of mushroom tyrosinase was analyzed. Pure protein, 0.1 to 0.3 mg, was hydrolyzed 22 or 72 hours at 110°C in 2 ml of constant boiling HCl, in evacuated, sealed tubes. The hydrolysates were analyzed on the Beckman model 120B and 116 analyzers by the standard methods. Half-cystine was determined by the method of Moore (24), and tryptophan by the method of Edelhoch (25).

**Sedimentation Velocity and Sedimentation Equilibrium**

These experiments were performed, and the results analyzed by the procedures of Applebury and Coleman (27). The partial specific volume used was 0.758 (5) for the native protein and 0.736 (calculated from the amino acid composition) for the protein in guanidine-HCl.

**Circular Dichroism**

Circular dichroism was measured with a Cary model 60 spectropolarimeter equipped with the circular dichroism attachment as previously described (28). Circular dichroism is expressed as molecular ellipticity, $[\theta] = 2.303 (4500 / \pi) (cL - cR)$. Molecular ellipticity is calculated on the basis of the moles of amino acid present.

**Treatment of Kinetic Data**

All kinetic data were subjected to statistical analysis with the use of programs written for the Hewlett-Packard model 9100A calculator. The method of Wilkinson (29) was used to estimate values of $K_m$ and $k_{cat}$, with their standard errors. Weighting factors used in the analyses were the inverse squares of the standard errors. Values of $K_{cat}$ of ionizing groups involved in the control of enzyme rates and $K_m$ values were determined by weighted least squares fits of plots of $K_m/k_{cat}$ or $1/k_{cat}$ versus $[H+]^2$ to straight lines.

**RESULTS**

**Purification of Enzyme**

Polyphenol oxidase, catalogue code TY, was purchased from Worthington. Several lots were used. The preparation as delivered was a lyophilized grey powder which was kept below 0°C until used, and had an activity which varied between 7 and 21 D.L-dopa units per mg.

Powder (500 mg) was stirred 1 hour with 25 ml of 0.1 M sodium phosphate, pH 7.0, 4°C. Solid ammonium sulfate, 5.38 g (Mann enzyme grade), was then added and stirring continued for 1 hour. This addition brought the ammonium sulfate concentration to 0.36 of saturation (calculated for 25°C). The precipitate was removed by centrifuging 1 hour at 27,000 rpm in the C30 head, Spinco model L preparative ultracentrifuge, 4°C. Solid ammonium sulfate, 2.06 g, was added to the supernatant, and the mixture was stirred 1 hour and centrifuged as before. The precipitate, containing 90 ± 10% of the starting units, was dissolved in about 4 ml of 0.1 M sodium phosphate, pH 7.0. This solution was treated three times with 1-ml portions of calcium phosphate gel (Bio-Rad, solids 80 mg per ml at pH 6.4), centrifuging off the gel each time in a clinical centrifuge and saving the supernatant. This treatment removed much of the black-brown pigment present in the crude extracts, with no loss in enzyme activity. Large amounts of this pigment were found to prevent sharp elution of the enzyme from DEAE-cellulose in the next step.

The partly decolorized enzyme solution was passed through a column (2.0 × 30 cm) of Sephadex G-25 “coarse,” equilibrated in 0.01 M sodium phosphate, pH 7.0, and the colored fractions, containing the enzyme, were applied directly to a column (2.0 × 15 cm) of DEAE-cellulose (Whatman DE52, previously swollen, microgranular), equilibrated with the same buffer. This column was eluted with a linear gradient of 0 to 0.3 M NaCl in starting buffer, total volume 400 ml. Fractions, 5 ml each, were collected at a rate of about six fractions per hour.

Small amounts of 280 mg absorbable material eluted during the first half of the gradient. These fractions contained no tyrosinase activity. The active material eluted sharply at about 0.15 M NaCl, but trailed into the leading edge of pigment, yellow in these dilute solutions. Most of the pigment remained at the top of the column. Fractions having a ratio OD$_{280}$/OD$_{450}$ greater than 1.4 were pooled, and concentrated by vacuum dialysis through cellophane bags (Schleicher and Schuell, Keene, New Hampshire), a procedure which gave stable solutions containing greater than 1 mg per ml of protein and resulted in no activity loss.

Over-all yields based upon recovery in the final step of units present in the crude extract were 25 ± 5%. The total recovery of units eluted across the entire gradient was 60 to 80% of those applied. Specific activities of the pooled fractions were about 420 to 450 dopa units per mg, derived from starting material containing 15 to 21 dopa units per mg. Starting material of lower specific activity (7 to 15 dopa units per mg) led to a purified preparation which was also of lower specific activity...
Mushroom Tyrosinase

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(240 to 360 dopa units per mg). It is therefore likely that the method of purification reported here is unable to separate inactivated molecules of tyrosinase from those which retain their activity.

Homogeneity of Preparations and Molecular Weight—Many previous studies have shown that preparations of tyrosinase from mushrooms, as well as those from some other sources, may be separated by such techniques as hydroxylapatite chromatography (6, 7) and starch gel electrophoresis (30) into several enzymatically active species. When our preparations were subjected to electrophoresis through polyacrylamide gels containing 8 M urea, they showed a single clear-cut band of rather high mobility, which appears to be the monomeric enzyme. In gels containing 0.08% sodium dodecyl sulfate, however, the protein hardly penetrated the gels at all, suggesting that it had aggregated.

In the ultracentrifuge at 60,000 rpm with scanner optics, our preparation sedimented as a single boundary. At 0.13 mg per ml, the $s_{20, w}$ was 5.76 S. This is somewhat lower than the values of 6.7 reported by Jolley, Robb, and Mason (31) at 7 mg per ml, 6.4 by Mallette and Dawson (4), and 6.3 reported by Kertész and Zito (6). The lower number which we obtained probably indicated that our preparation was more extensively dissociated than those studied by these other authors. This impression was confirmed by high speed equilibrium ultracentrifugation measurements with the short column technique of Yphantis (32). The pattern obtained from one preparation is shown in Fig. 1. The lowest molecular weight observed corresponded to about 32,000, and the highest to about 118,000.

In 5.8 M guanidine-HCl, however, a single species was obtained which represents a homogeneous monomeric species of apparent molecular weight ~26,000 (Fig. 1). It has generally been assumed that the molecular weight of the monomer of mushroom tyrosinase is near 32,000 (33,34) and the line drawn in the upper part of Fig. 1 represents a molecular weight of 32,000. We currently do not have an explanation for the lower value found in guanidine-HCl unless the assumption of the calculated partial specific volume is incorrect. Because of this uncertainty we have continued to use the accepted monomer weight of 32,000 for calculations of specific activity.

Absorption Spectrum and Optical Activity—The optical density of a 1% solution of the present preparation of mushroom tyrosinase at 281 mp, the absorbance peak, was 24.9 ± 0.3, when examined immediately after purification. This number is fully explained by the tyrosine and tryptophan content of the enzyme, as Kertész has remarked (34). Occasional increases above this value reflect the degree to which tyrosinase has undergone self-oxidation. The ratio of $A_{290}:A_{260}$ was normally 0.0235 to 0.0605, and that for $A_{260}:A_{250}$ was 1.51 to 1.62. As preparations were stored over a period of a few months, however, there was gradual development of a shoulder at 340 mp, and a lowering of the $A_{340}:A_{260}$ ratio. Both effects are known to occur in the spectra of proteins which are being oxidized by tyrosinase (35).

Ultraviolet circular dichroism of native tyrosinase is shown in Fig. 2. The CD spectrum is unusual, in that the negative ellipticity band at about 220 mp is markedly less deep than that at 206 mp. The CD spectrum also shows weak positive ellipticity bands of $[\theta]$ per residue about 100° in the region of aromatic side chain absorption. This corresponds to a molar ellipticity (per monomer) of $[\theta] \approx 30,000.$

Several small samples of mushroom tyrosinase were dialyzed...
overnight in the cold against 0.05 M sodium acetate-0.05 M sodium phosphate, at pH values of 2, 3, 4, 5, 6, and 7. The CD spectra of these samples were measured in the spectral range 190 to 250 nm (Fig. 2). The gross conformation of the protein, as measured by CD, does not change detectably between pH 7 and 4, the range over which the kinetic results described below were carried out. At lower pH there is a shift to a new pattern with a maximum trough depth at 211 nm. Overnight dialysis of the pH 2 and 3 samples against pH 7 buffer failed to restore the changes; thus, irreversible under these conditions, unlike those which occur when a protein-like Escherichia coli alkaline phosphatase is adjusted to low pH (27).

**Amino Acid Composition**—Jolley et al. (31, 33) and Zito and Kertész (36) have recently presented complete amino acid analyses of purified preparations of mushroom tyrosinase. These are in reasonable agreement with each other, and with preliminary data published earlier by Bouchilloux, McMahl, and Mason (7). Our own analyses, conducted on six different preparations of the enzyme, are summarized in Table I. These results are reproducible to about ±5% between preparations. They agree in general with those just referred to, except that we find 12 residues of tryptophan per 32,000 molecular weight, rather than the values between 7 and 11 reported by Jolley et al. (31, 33) and Zito and Kertész (36), respectively. The difference in results possibly arises from differences in techniques of analysis.

**Copper Content**—One mole of copper per 32,000 molecular weight corresponds to a copper content of 0.199%. Values measured by atomic absorption analysis of several of our preparations are given in Table II. From these it is clear that our samples of enzyme, like those of others (4, 5, 7, 31, 37), contain somewhat less than 1 mole of copper per mole of monomer. Kertész (38) has reported that all or most of the copper in mushroom tyrosinase reacts chemically as Cu(I), although some oxidation accompanies aging of the preparations. Similar observations have been made by others (7, 39), using electron spin resonance to measure the enzymic content of Cu(II). Generally, some Cu(II) is found, and this may well be the catalytically inactive copper that Kubowitz first suggested to be present in some preparations of the potato enzyme (8).

**Oxidation of Series of Para-Substituted Catechols by Tyrosinase**—In order to determine some of the chemical features influencing the oxidation of the catechol molecule by tyrosinase, a series of catechols were prepared containing substituents in position 4 which are electron-withdrawing with respect to hydrogen in the order H < SCN < COCH₃ < CHO < CN < NO₂. Substitution at position 4 was chosen to minimize steric interactions with the substrate hydroxyl groups. Catechols substituted with electron-releasing groups in position 4 could not be used because the catechols either gave highly unstable products (4-methyl-, 4-aminocatechol) or behaved anomalously as substrates (4-hydroxycatechol, expected to yield a mixture of tautomers of hydroxyquinones upon oxidation).

Spectrophotometric assays for these substituted catechols were developed by determining the position and molar extinction coefficients of the spectral peaks characteristic of the quinone oxidation products. Since the general methods for the synthesis of o-benzoquinones (40, 41) are unsuitable for compounds containing strongly electron-withdrawing substituents, the spectra of the quinones were obtained by following spectrophotometrically the total oxidation of known amounts of the catechols by large amounts of tyrosinase. A series of spectra collected during the total oxidation of 4-nitrocatechol by tyrosinase is shown in Fig. 3 and clearly indicates that only two species are present. All the resultant quinones are stable for at least 30 min before further spectral changes occur. The positions of the absorption maxima and the molar extinction coefficients of the series of quinones corresponding to the above series of catechols are given in Table III, and were used to determine the molar rates of oxidation of the catechols in the kinetic experiments. All of these catechols are oxidized relatively slowly compared to pyrocatechol, do not produce reaction inactivation of the enzyme, and give linear progression curves. Thus initial velocities can be read directly from the optical density tracings without the extrapolation necessary for the pyrocatechol assay.

Values of $k_{cat}$ and $K_m$ at pH 7.02 in air (20.95% O₂) for this series of catechol substrates are given in Table IV. Hammett plots of the data are shown in Figs. 4 and 5 with $σ^-$ values given...
OXIDATION OF 4-NITROCATECHOL BY TYROSINASE

**FIG. 3.** Oxidation of 4-nitrocatechol by mushroom tyrosinase. The starting mixture contained 2.50 ml of 50.6 \( \mu \)M 4-nitrocatechol in 0.1 M sodium phosphate, pH 7.38, in a cuvette of 1-cm path length. After an initial scan in the Cary model 15 (Curve 1), 62 \( \mu \)g of tyrosinase were added in 0.50 ml of buffer, and successive scans were run at the time intervals shown. Curve 10, for infinite time, was that obtained at 150 and also 250 min after adding enzyme. Curve 1 is corrected for the 6:5 dilution factor on adding enzyme.

**TABLE III**

\( \lambda_{\text{max}} \) and \( \epsilon_M \) values used in assays of catechol oxidations

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( \lambda_{\text{max}} )</th>
<th>( \epsilon_M )</th>
</tr>
</thead>
<tbody>
<tr>
<td>l-Dopa</td>
<td>475  ( \pm ) 5</td>
<td>3600  ( \pm ) 5</td>
</tr>
<tr>
<td>Pyrocatechol</td>
<td>390</td>
<td>1830</td>
</tr>
<tr>
<td>4-Thiocyanato-</td>
<td>395</td>
<td>1330</td>
</tr>
<tr>
<td>4-Acetyl-</td>
<td>410</td>
<td>1090</td>
</tr>
<tr>
<td>4-Cyano-</td>
<td>430</td>
<td>2880</td>
</tr>
<tr>
<td>4-Formyl-</td>
<td>410</td>
<td>1470</td>
</tr>
<tr>
<td>4-Nitro-</td>
<td>388.5</td>
<td>8500</td>
</tr>
</tbody>
</table>

a Refers to formation of dopachrome; all others refer to quinone formation.

Quinone peak is at 380 \( \mu \)M, \( \epsilon_M = 15,400 \). Since 4-nitrocatechol dissociates 1 proton with a \( pK \) of about 6.5 and an accompanying change in spectrum occurs, the wave length isoelectic for the protonated and deprotonated forms, 388.5 \( \mu \)M, was used here.

**FIG. 4.** Hammett plot of values of log \( K_m \) for oxidation of catechol substrates as a function of the substituent parameter \( \sigma^+ \). The points are experimental points, measured for compounds of the structures shown adjacent to those points. ---, the least squares line for the four compounds whose structures appear on the upper side of the figure; ------, the least squares line for all six data points.

**TABLE IV**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( k_{\text{cat}} )</th>
<th>( K_{\text{cat},\text{catechol}} )</th>
<th>( K_{\text{O}_2} )</th>
<th>( K_{\text{cat},\text{O}_2} )</th>
<th>( K_s ) for ( \text{O}_2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>l-Dopa</td>
<td>221 ( \pm ) 2</td>
<td>26.3 ( \pm ) 0.9</td>
<td>14.0 ( \pm ) 0.64</td>
<td>3.21 ( \pm ) 0.94</td>
<td>1.65 ( \pm ) 0.50</td>
</tr>
<tr>
<td>Pyrocatechol</td>
<td>1049 ( \pm ) 25</td>
<td>19.39 ( \pm ) 1.40</td>
<td>1.89 ( \pm ) 2.12</td>
<td>1.39 ( \pm ) 0.78</td>
<td>1.72 ( \pm ) 1.01</td>
</tr>
<tr>
<td>4-SCN</td>
<td>0.02 ( \pm ) 4.1</td>
<td>8.10 ( \pm ) 1.44</td>
<td>8.63 ( \pm ) 0.40</td>
<td>4.99 ( \pm ) 0.50</td>
<td>28.2 ( \pm ) 3.21</td>
</tr>
<tr>
<td>4-COCH_3</td>
<td>6.43 ( \pm ) 0.17</td>
<td>2.74 ( \pm ) 0.29</td>
<td>1.41 ( \pm ) 1.42</td>
<td>1.58 ( \pm ) 0.94</td>
<td>6.37 ( \pm ) 3.81</td>
</tr>
<tr>
<td>4-CHO</td>
<td>2.12 ( \pm ) 0.07</td>
<td>1.53 ( \pm ) 0.20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-CN</td>
<td>0.0265 ( \pm ) 0.0015</td>
<td>1.37 ( \pm ) 0.21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-NO_2</td>
<td>0.0241 ( \pm ) 0.0020</td>
<td>0.427 ( \pm ) 0.112</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( \epsilon_M \) for oxygen is calculated on the assumption that \( \text{O}_2 \) is the first substrate to bind; \( K_s \) is then the ratio \( K_{\text{cat},\text{O}_2}/K_{\text{cat},\text{catechol}} \). The ordered bisubstrate rate equation was used to fit the data obtained when oxygen was varied as well as catechol (42). In the double reciprocal form this is as follows.

\[
\frac{1}{v} = \frac{1}{V} \left( 1 + \frac{k_{\text{cat}}}{[\text{cat}]} + \frac{K_{\text{O}_2}}{[\text{O}_2]} + \frac{K_s K_{\text{cat}}}{[\text{cat}][\text{O}_2]} \right)
\]
Hammett plots except that for $k_{cat}$ when all six substrates are included have correlation coefficients greater than 0.95.

The temperature dependence of the rate of oxidation of three of these catechols (pyrocatechol, 4-acetyl-, and 4-formylcatechol) by mushroom tyrosinase was determined over the range 0–30°. The energies of activation, obtained from Arrhenius plots of $k_{cat}$, are given in Table VB. The reaction rate becomes highly temperature-dependent as the electron-withdrawing character of the substrate substituent increases. An isokinetic relationship (43) exists between $\Delta H^\ddagger$ and $\Delta S^\ddagger$ yielding a value of $\beta$ of 421° ± 36° K, which is within the range found for a great many chemical reactions.

**Initial Velocity Patterns with Oxygen Varied—Oxidation of catechols in air fixes one of the cosubstrates, O$_2$, at 20.95%. In order to determine the region of oxygen saturation of the enzyme**

![FIG. 6](http://www.jbc.org/)

**FIG. 6. Oxidation of pyrocatechol (A), 4-acetylcatechol (B), and 4-formylcatechol (C) by mushroom tyrosinase, as a function of concentrations of catechol and oxygen.** The volume percentage of oxygen in the gas phase is given beside the appropriate curve. Lines are drawn according to the results of the least squares fitting procedures described under “Materials and Methods.”

---

**TABLE V**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{cat}$ (kcal/mole)</th>
<th>$\Delta H^\ddagger$ (kcal/mole)</th>
<th>$\Delta S^\ddagger$ (cal/K mole)</th>
<th>$\Delta G^\ddagger$ (kcal/mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrocatechol</td>
<td>2.19 ± 0.69</td>
<td>1.00 ± 0.69</td>
<td>39.3 ± 2.3</td>
<td>13.33</td>
</tr>
<tr>
<td>4-COCH$_3$</td>
<td>11.14 ± 0.89</td>
<td>10.55 ± 0.87</td>
<td>-20.0 ± 2.9</td>
<td>16.52</td>
</tr>
<tr>
<td>4-CHO</td>
<td>16.35 ± 1.17</td>
<td>15.70 ± 1.17</td>
<td>-4.4 ± 3.9</td>
<td>17.09</td>
</tr>
</tbody>
</table>

* The value of $E_{cat}$ for $K_m$ for pyrocatechol is 4.94 ± 0.97 kcal per mole.
in the presence of these various catechols and to determine the nature of the dependence of the enzymatic rate on oxygen concentration, a series of initial rate measurements was made as a function of catechol concentration at various fixed levels of oxygen. The results in double reciprocal form are shown in Fig. 6. A series of parallel or very slightly convergent lines is obtained in the case of pyrocatechol (Fig. 6A). Marked substrate inhibition complicates the picture at high pyrocatechol and low oxygen concentrations. On the other hand a series of converging lines is obtained in the case of acetylcatechol, and substrate inhibition does not occur (Fig. 6B). Similar features are shown by 4-formylcatechol (Fig. 6C). When the data for the various substrates are fitted to the steady state initial rate equation for the ordered bisubstrate mechanism, the values of the Michaelis constants for catechol and oxygen obtained are those given in Table IV. A concentration of oxygen as low as 2.8% in the gas phase barely desaturates the enzyme in the presence of 4-thiocyanato-catechol.

**pH Dependence of Michaelis Parameters for Catechol Oxidations**

—Previous qualitative surveys of the pH dependence of the oxidation of pyrocatechol and dopa by tyrosinase have revealed a relatively constant activity between pH 5.5 and 8.0 and a decrease in rate at lower pH values (9, 22, 45, 46). Assays at higher pH are unreliable because of instability of both substrates and the quinone products. Values of $K_m$ and $k_{cat}$ between pH 3.8 and 7.5 for pyrocatechol, 4-acetyl-, and 4-formyl-catechol are given in Table VI. Each value represents an experiment containing 9 to 12 points. In each case the $K_m$ data were satisfactorily fit by a single sigmoid titration curve. Plots of the data obtained for pyrocatechol and 4-formylcatechol are given in Fig. 7.

<table>
<thead>
<tr>
<th>pH</th>
<th>$K_m$ (M$^{-1}$)</th>
<th>$k_{cat}$ (sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.02</td>
<td>0.0222 ± 0.022</td>
<td>1230 ± 50</td>
</tr>
<tr>
<td>6.06</td>
<td>0.238 ± 0.048</td>
<td>1130 ± 90</td>
</tr>
<tr>
<td>5.08</td>
<td>0.284 ± 0.019</td>
<td>1040 ± 30</td>
</tr>
<tr>
<td>4.51</td>
<td>0.740 ± 0.245</td>
<td>1290 ± 190</td>
</tr>
<tr>
<td>4.15</td>
<td>2.12 ± 0.28</td>
<td>908 ± 78</td>
</tr>
<tr>
<td>3.82</td>
<td>2.61 ± 0.39</td>
<td>1290 ± 120</td>
</tr>
</tbody>
</table>

*In the case of pyrocatechol, $k_{cat}$ was invariant with pH in the region studied.*

---

**Table VI**

<table>
<thead>
<tr>
<th>pH</th>
<th>$K_m$ (M$^{-1}$)</th>
<th>$k_{cat}$ (sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.02</td>
<td>0.0222 ± 0.022</td>
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<td>6.06</td>
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<td>1290 ± 190</td>
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<td>4.15</td>
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<td>908 ± 78</td>
</tr>
<tr>
<td>3.82</td>
<td>2.61 ± 0.39</td>
<td>1290 ± 120</td>
</tr>
</tbody>
</table>

---

**Fig. 7.** Plots showing log $k_{cat}$, log $K_m$, and log ($k_{cat}/K_m$) as functions of pH, for the oxidation of pyrocatechol (A) and 4-formyl-catechol (B). The points are experimental points, obtained by fitting procedures as described under “Materials and Methods,” and listed with their standard errors in Table VI. ---, theoretical curves, assuming the ionizations of single protons with the pH values shown in the figure and in Table VI; ----, tangents to the theoretical curves, having slopes of 0 or ±1, which intersect at values of pH corresponding to the pH values of acid dissociable groups which are kinetically significant.
Fig. 8. Kinetic patterns of inhibition of tyrosinase. A, inhibition by benzoic acid with respect to pyrocatechol. Succinate-pyrophosphate buffer, pH 5.0. Concentrations of benzoic acid were 0, 0.749, 2.00, and 3.00 μM. B, inhibition by benzoic acid with respect to oxygen. Benzoic acid concentrations were 0, 1.87, 2.74, and 3.01 μM. C, inhibition by cyanide with respect to catechol. Phosphate buffer, 0.1 M, pH 8.0. Concentrations of NaCN were 0, 16.6, and 49.9 μM. D, inhibition by cyanide with respect to oxygen. Phosphate buffer, 0.1 M, pH 8.0. Concentrations of NaCN were 0, 17.7, 53.1, and 106 μM. For experiments in which catechol was varied, the gas phase was air; for those in which oxygen was varied, the catechol concentration was 0.20 mM. In D, the assumption was made in drawing the lines that the pattern showed competitive inhibition. In the other cases, least squares fits were used as described under "Materials and Methods."

According to the method recommended by Dixon (47). The pKₐ values determined by the weighted least squares fit (see "Materials and Methods") are given in Table VI. Kₐ values for all three catechols show dependence on an ionization with a pKₐ between pH 4.9 and 6. This pKₐ is significantly higher in the case of the two substituted catechols. An additional lower pKₐ may be involved as indicated by the kₐₐ data for 4-acetyl- and a 4-formylcatechol.

Kinetics of Inhibition of Tyrosinase by Benzoic Acid and Cyanide —The nature of the inhibition exerted by benzoic acid on the catecholase activity of tyrosinase is shown by the kinetic results in Fig. 8. These are double reciprocal plots of data obtained with
TABLE VII

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Nature of inhibition</th>
<th>Nature of inhibition</th>
<th>Oxygen varied</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>Competitive</td>
<td>1.02 ± 0.17</td>
<td>2.96 ± 0.14</td>
</tr>
<tr>
<td>Cyanide</td>
<td>Noncompetitive</td>
<td>14.7 ± 2.0</td>
<td>7.83 ± 0.23</td>
</tr>
</tbody>
</table>

a Obtained with the assumption that the data in Fig. 8D show a competitive inhibition.

**Fig. 9.** Binding of [14C]-benzoic acid to mushroom tyrosinase, by equilibrium dialysis. Double reciprocal plot. Succinate-pyrophosphate buffer, pH 5.0. Enzyme was 2.46 µM in tetramer. For other details see "Materials and Methods." The values obtained by least squares fitting of the data to a hyperbola, as total binding sites ([enzyme]_{tot}) and dissociation constant (K_i) are given in the graph.

The binding constant obtained from this data is 1.9 x 10^{-6} M compared to 1.02 x 10^{-6} M for K_i obtained from the inhibition data in Fig. 8A. Maximum stoichiometry of binding observed, however, was between 1 and 2 molecules of benzoic acid per tetramer of tyrosinase (Table I), and was quite variable from preparation to preparation. The close agreement between the kinetic and binding data show that at the concentration of benzoic acid where the maximum stoichiometry was observed the enzyme was completely inhibited (see "Discussion").

**Catechol Activation of Oxidation of Tyrosine—L-Tyrosine is the best phenol substrate of tyrosinase. Catechols in low concentrations have been observed to be activators of the phenolase activity of tyrosinase (49-51). The progress curve for the oxidation of L-tyrosine is characterized by a lag time, the overcoming of which has been satisfactorily explained by the fact that the dopa, an activator of tyrosine oxidation, is the immediate product of that oxidation (52). Recently Pomerantz and Warner (53), using the hamster enzyme, found that the lag time was shortened by added L-dopa in such a way as to suggest that the dopa was saturating a specific binding site on the enzyme. The apparent K_m found for the activation, 2 x 10^{-6} M, was two orders of magnitude smaller than the K_m of L-dopa as a substrate. With the mushroom enzyme the reciprocal of the lag time, \( 1/T_{lag} \), defined according to Lerner et al. (49), is a linear function of the enzyme concentration (Fig. 10).

Duration of the lag time has also been observed to be dependent on the concentration of phenol substrate (51, 54). In order to determine this effect quantitatively for mushroom tyrosinase we have measured the lag time as a function of activator (L-dopa) concentration at various fixed concentrations of L-tyrosine (Fig. 11). The method of plotting the data in Fig. 11 is according to the method of Pomerantz and Warner (53). By assuming that dopa saturates an "activation site" according to a simple binding isotherm and that the lag time is proportional to the amount of enzyme still not "activated" at zero time, they reason that

\[
\frac{1}{T} = \frac{1}{T_0} + \frac{1}{T_m} [\text{dopa}]
\]

K_m is the apparent Michaelis constant for activation, and T is the lag time in the absence of added dopa, while \( T_0 \) is the lag time when a given concentration of dopa has been added.

From Fig. 11 it is seen that T, the "intrinsic" lag time, is prolonged by increasing concentrations of L-tyrosine, or expressed in terms of the plots in Fig. 11, increasing concentrations of L-dopa are required for equal shortening of the lag time in the face of increasing concentrations of L-tyrosine. Extrapolation of the apparent K_m for L-dopa as activator (calculated from the...
FIG. 10. Relationship between the lag time for L-tyrosine oxidation and enzyme concentration. The reciprocal of $t_{lag}$, in seconds, is plotted against the concentration of enzyme, measured as the activity ($\Delta$O.D.₃₆₅ per sec) finally achieved when the lag is over. A final activity of 0.01 $\Delta$O.D.₃₆₅ per sec corresponds to 0.142 µg of tyrosinase under these conditions. The points are experimental points; the line is the unweighted least squares line.

FIG. 11. Plots of reciprocals of $t_{lag}$ as a function of L-dopa concentration at different concentrations of L-tyrosine. The explanation for this type of plot is given in the text.

data in Fig. 11) to zero tyrosine concentration gives a value of $4.7 \times 10^{-4}$ M, over two orders of magnitude less than the $K_m$ for L-dopa acting as a substrate (Table IV).

DISCUSSION

The preparations of mushroom tyrosinase described in this paper are similar to those reported in previous investigations (3-7). There are several indications that these samples of purified enzyme contain inactivated molecules. The copper content of the enzyme is variable as is the stoichiometry of benzoic acid binding (Table II). Binding of less than 2 moles of benzoic acid per mole of tetramer in each case, however, totally inactivates the enzyme (Figs. 8 and 9). Thus all sites contributing to the activity have interacted with inhibitor. The stoichiometry for total copper content would appear to approach 1 g atom per monomer of $\sim$30,000 daltons or 4 g atoms per mole of tetramer, although the average value for five of the present preparations is $3.2 \pm 0.23$ (standard deviation) with a range of 2.8 to 4.4 suggesting loss of total copper in most cases.

If benzoic acid binding is adjusted upward by assuming that binding is lost in direct proportion to the reduction of copper content below 4 g atoms per tetramer, then the maximum stoichiometry obtained is 1.82 moles of benzoic acid per mole of tetramer, suggesting that there may be two binding sites for benzoic acid per tetramer (one per dimer) or two per 4 atoms of copper. Total removal of copper by cyanide does result in total loss of benzoic acid binding (Table II).

Precise interpretation of the stoichiometry is difficult since the degree of dissociation of the protein may possibly affect both activity and inhibitor binding (see Fig. 1), and the presence of Cu(III) rather than Cu(I) might interfere with inhibitor binding. Most preparations of tyrosinase are reported to contain at least some Cu(II) which may be inactive (38, 39). It is in fact possible that in some cases more than half of the molecules are inactive, and that one active site is present per monomer in completely undamaged tyrosinase. The present results make less likely any mechanistic hypothesis involving 4 copper atoms per active site, or one per tetramer, but do not allow a definite choice between 2 atoms or 1 of copper per active site.

Both the secondary and tertiary structure of the protein appear to be stable over the pH range 7 to 4 as judged by the invariance of the ultraviolet circular dichroism (Fig. 2). The CD spectrum includes a contribution from the aromatic chromophores of the protein which must reflect the environment conferred by the tertiary folding of the molecule. The deep ultraviolet CD spectrum is difficult to interpret on the basis of a combination of the CD spectra of known conformations of the polypeptide chain. There does appear to be a negative band near 222 nm suggesting the presence of $\alpha$ helix, although an unexpectedly large negative band appears at 205 to 208 nm. There may be contributions from low ultraviolet aromatic chromophores. Unfortunately high absorbance of the protein prevents meaningful measurement of circular dichroism below 200 nm. It is of interest that recent computer generation of the CD curves expected for a protein containing 60% random structure with the remainder of the peptide chain present largely as $\alpha$ helix, or $\alpha$ helix and a small amount of $\beta$ structure, result in spectra rather similar to that observed for tyrosinase above 200 nm (55).

The inhibition kinetics of benzoic acid and cyanide acting as inhibitors of the catecholase reaction (Fig. 8) suggest that two separate substrate-binding sites exist within the active center, one for catechol and one for oxygen. Benzoic acid competes for the catechol site, while cyanide interacts with the oxygen site (Table VII). The latter appears to involve the copper ion. Tyrosinase is not a favorable subject for the standard approaches of steady state kinetics, since the reactions are functionally irreversible and the products are either unstable (the o-quinones) or are substrates for the enzyme (the catechols). With the use of freshly prepared $o$-benzoquinone, we have ascertained that
this compound exerts no detectable inhibition on pyrocatechol oxidation at a concentration of 1 mM.

In order to be a good substrate the catechol must maintain a density of readily removed electrons around the —OH groups at positions 1 and 2 of the ring. Progressive withdrawal of these electrons markedly slows the oxidation (Table IV, Fig. 5). Four of these catechols obey a satisfactory Hammett relationship for both \( K_m \) and \( k_{cat} \) (Table VA, Figs. 4 and 5), while pyrocatechol, 4-acetyl-, and 4-formylcatechol show a satisfactory isokinetic relationship for \( k_{cat} \) (Table VB). Thus these substrates must be oxidized by the same basic mechanism; i.e. the rate-limiting step is not altered by the change in substrate. As electrons are withdrawn to the para substituent, binding of the catechol becomes tighter, since saturation of the enzyme occurs at progressively lower substrate concentrations (Table IV). For the two catechols, 4-cyano- and 4-nitrocatechol, which deviate from the Hammett relationship, the most severe deviation occurs in the plot of \( k_{cat} \) (Fig. 5). Both substrates, however, are bound at least an order of magnitude more tightly than pyrocatechol suggesting that it is the rate of electron transfer rather than the substrate-binding function which is abnormal. Since the \( \rho \) value for \( k_{cat} \) is more negative than that for \( K_m \), more than one rate constant must be altered by the substituent in these substrates.

Initial velocity data on the oxidation of this series of catechols as a function of oxygen saturation provide some useful information regarding the mechanism of action of this enzyme. The double reciprocal plot in Fig. 6A, referring to the oxidation of pyrocatechol, seems to consist of a series of parallel lines, although the poor precision of the data at the lowest oxygen concentrations and the profound substrate inhibition by catechol prevent certainty on this point. Such a pattern is expected for ping-pong mechanisms (42), but is also seen for certain sequential mechanisms in which the rate of turnover of the enzyme is much faster than the rate of dissociation of the first enzyme-substrate complex formed (56). That a sequential mechanism is involved is indicated by the pattern of intersecting lines seen in the double reciprocal plots in Fig. 6, B and C, referring to the oxidation of 4-acetyl- and 4-formylcatechol. Since these substrates are oxidized much more slowly than pyrocatechol (Table IV), it is reasonable that the intersecting pattern should be revealed in these cases.

Ingraham (57) has investigated the variation of the Michaelis constant for oxygen \( (K_{O2}) \) with catechol concentration, using three different catechol substrates and a tyrosinase prepared from French prunes. His results are consistent with a sequential mechanism and he found that \( K_m \), the limiting value of \( K_{O2} \) as the substrate went to zero, was the same for the three catechols studied. This observation was taken to mean that oxygen is the first substrate bound by tyrosinase (57). If this assumption is correct, then we may estimate \( K_m \) for oxygen for the substrates studied in Fig. 6 (Table IV). It can be seen that the values of \( K_m \) as a function of the catechol vary widely. By extending investigations to substrates much less rapidly oxidized than those studied by Ingraham (57), we have shown that \( K_m \) for oxygen is actually not independent of the catechol substrate used. This implies that if the reaction is ordered, oxygen does not bind first.

One molecule of oxygen oxidizes 2 molecules of reducing substrate in the reactions catalyzed by tyrosinase (1). The reactions are therefore of 1st reactivity, in Cleland’s terminology (42), and it is necessary to include a 2nd molecule of catechol in any mechanistic scheme for catecholase activity. At present there is no evidence as to how this should be done. Catechols are well known activators of the hydroxylase activity of tyrosinase (49, 52, 53), at concentrations near micromolar (Fig. 11). It may be that catechol oxidation is likewise activated by catechol; however, this would occur at micromolar substrate concentrations. We have searched for a departure from Michaelis-Menten kinetics in catechol oxidation at micromolar levels of substrate. While apparent deviations are observed, the precision of rate measurements under these conditions is too low to allow definite conclusions to be drawn.

Tyrosinase does compete with dopa for the activator site (Fig. 11). The competition is not effective, however, except at high concentrations of dopamine or greater, while the true \( K_m \) of dopa for the activator site is \( 0.47 \times 10^{-4} \text{ M} \). Thus dopa is far more specific for this site. This relationship would be compatible with a mechanism in which the initial hydroxylase mechanism shifts to one activated by catechol as soon as micromolar concentrations of the product catechol appear. Such a shift (expressed by the lag time) would be expected to be inversely proportional to enzyme concentration as is observed (Fig. 10).

These observations extend the list of inhibition effects exerted by catechol on phenol oxidation (13, 58) and vice versa (13). It is still far from clear, however, whether both the catecholase and the phenolase activities of tyrosinase are functions of a single active site. All the kinetic observations certainly could be incorporated into such a unified mechanism. It is noteworthy that catechols are activators of phenol hydroxylation, and that a 2nd molecule of catechol, expected on stoichiometric grounds, is yet to be accounted for in the mechanism of catechol oxidation.

One further feature of the comparative kinetic measurements done on the various substituted catechols requires comment. The inflections in the log \( (k_{cat}/K_m) \) versus pH curves, which in unsubstrate mechanisms are to be interpreted as arising from proton dissociations in the free enzyme or substrate, occur at different positions for different substrates (Fig. 7, Table VI), although none of the substrates has a \( pK_a \) itself in this range. This substrate dependence of an enzyme \( pK_a \) could be explained if the enzyme form with which the "substrate" catechol combined already contained a molecule of catechol. This explanation is in agreement with the expectation that 2 catechol molecules are involved in the catecholase mechanism. Taken in conjunction with the other data presented in this paper, it would imply that a quaternary complex of enzyme, oxygen, and two catechols is formed during the oxidation of catechol by tyrosinase.

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Harry W. Duckworth and Joseph E. Coleman


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