Phytochemistry, 1966, Vol. 5, pp. 665 to 675. Pergamon Press Ltd. Printed in England

SUBSTRATES AND INHIBITORS OF THE ACTIVATED TYROSINASE OF BROAD BEAN (*VICIA FABA* L.)

D. A. ROBB,* T. SWAIN and L. W. MAPSON

Low Temperature Research Station, Cambridge

(*Received* 28 *July* 1965)

Abstract—The specificity and affinity of broad bean tyrosinase for phenolic substrates and inhibitors has been investigated. The Michaelis constant is smaller for the more simple derivatives of catechol than for DOPA and the dissociation constant for phenolic inhibitors has been found to be comparable to the Michaelis constants. The observation that two pH optima exist depending on whether an alkyl phenol or one containing a weakly ionizing carboxylic group is assayed has been interpreted on the basis that such a group when undissociated may chelate (depending on steric conditions) with the functional copper and thereby cause an inhibition. This theory has been extended to cover inhibition by inorganic anions, and evidence is presented that hydrogen fluoride and hydrazoic acid rather than the respective anions are responsible for the strong inhibition observed in the pH region 4·1–6·0. It is predicted from a kinetic study of the interdependence of enzymic activity on the concentrations of oxygen and *p*-cresol that the enzyme exists in two forms during its reaction sequence.

INTRODUCTION

TYROSINASE can conveniently be purified from extracts of broad bean leaves. The existence of four or more forms of the enzyme in such extracts was demonstrated electrophoretically[1] but no distinction was observed in substrate specificity. Thus the heterogeneity in this tyrosinase resembles that found in the enzyme from *Neurospora crassa*[2] rather than that reported for mushroom *Psalliota campestris* or *Agarious bispora*,[3,4] where the forms can be differentiated by the relative rates at which monohydroxy- and orthodihydroxy-phenols are oxidized.

The activity of broad bean tyrosinase is enhanced by treatment with a wide variety of denaturing agents[5–7] and in this paper the properties of enzyme fractions activated by treatment with the anionic detergent sodium bis(2-ethylhexyl)sulphosuccinate (manoxal or aerosol OT) are reported. The first part of the communication concerns the characterization of the enzyme; in the second, some of its general properties are described and interpreted in accordance with current hypotheses concerning the mode of action of enzymes of this type.

RESULTS

The Substrate Specificity of Broad Bean Tyrosinase

Various phenols, all of which have been shown to be substrates of "phenol oxidases" (laccases, peroxidases, tyrosinases) from other sources, were assayed with broad bean and

* Present address: University of Oregon Medical School, Department of Biochemistry, 3181 S.W. Sam Jackson Park Road, Portland 1, Oregon, U.S.A.

[1] D. A. ROBB, L. W. MAPSON and T. SWAIN, *Phytochem.* **4**, 731 (1965).
[2] M. FLING, N. H. HOROWITZ and S. F. HEINEMANN, *J. Biol. Chem.* **238**, 2045 (1963).
[3] J. L. SMITH and R. C. KRUEGER, *J. Biol. Chem.* **237**, 1121 (1962).
[4] S. BOUCHILLOUX, P. MCMAHILL and H. S. MASON, *J. Biol. Chem.* **238**, 1699 (1963).
[5] R. H. KENTEN, *Biochem. J.* **67**, 300 (1957).
[6] R. H. KENTEN, *Biochem. J.* **68**, 244 (1958).
[7] D. A. ROBB, L. W. MAPSON and T. SWAIN, *Nature* **201**, 503 (1964).

potato tyrosinases at pH 5·0 and the results are presented in Table 1. For comparison the activities are related to that shown toward DL-β-(3,4-dihydroxyphenyl)alanine (DOPA): although certain other phenols are oxidized more rapidly (cf. Table 1), this compound was chosen as the standard because with it, reaction inactivation[8] of the broad bean enzyme was small. The Michaelis constants for broad bean tyrosinase are relatively high varying, for example, from 1·0 to 14 mM for caffeic acid and DOPA respectively (see Table 3), and therefore the data given in Table 1 for DOPA and other phenols of similar molecular weight

TABLE 1. RELATIVE RATES OF OXIDATION OF SUBSTRATES OF BROAD BEAN LEAF TYROSINASE

Diphenol substrates	Relative rate*	Monophenols	Relative rate*
Homocatechol	400–450	*p*-Cresol	8†
Catechol	180–200	Tyramine	2†
Pyrogallol	170–190	*p*-Coumaric acid	0·1†
DOPA	100	*Others*	
Adrenaline	100†	Ascorbic acid	0
Caffeic acid	25	*p*-Phenylenediamine	0
Chlorogenic acid	16		
Gallic acid	0·43†		
Protocatechuic acid	0·22†		

* Relative to DOPA equals 100. An enzyme purified 150-fold was used throughout with 4 mg of each substrate.

† Using the manometric procedure, the other data relates to the oxygen cell assay.

(e.g. chlorogenic acid) is based on measurements made when the enzyme was not fully saturated (the concentrations of homocatechol and DOPA employed were 10·6 mM and 6·7 mM respectively). Although the low solubilities of some acids in aqueous solutions preclude their use in amounts necessary for the enzyme to be saturated, it was shown that even when the theoretical maximal velocities (determined from the intercept of Lineweaver–Burk plots) for these substrates is employed, the order in which the phenols are oxidized is unchanged. Visual inspection of the colour changes associated with the action of the broad bean preparation on both tyrosine and DOPA indicated that the same red intermediate (dopachrome) and black precipitate (melanin) were formed—a result suggesting that tyrosine is initially converted to DOPA by hydroxylation. That the enzyme would catalyse hydroxylation of monophenols was confirmed by showing chromatographically that homocatechol was formed from *p*-cresol[9] when the latter oxidized in the presence of an excess of ascorbic acid.

These results confirm that the "phenol oxidase" of broad bean is a typical tyrosinase, able to catalyse the ortho-hydroxylation of monohydroxyphenols and the dehydrogenation of ortho-dihydroxyphenols. They also tend to emphasize the fact that within the bounds of this general substrate specificity each organism produces a tyrosinase with a distinctive substrate pattern varying from the relatively unspecific plant tyrosinases to mammalian tyrosinase which is virtually specific for DOPA and tyrosine.[10] The feature of the substrate

[8] J. M. NELSON and C. R. DAWSON, *Advances Enzymol.* **4**, 99 (1944).
[9] A. M. MAYER and J. FRIEND, *J. Exptl Bot.* **11**, 143 (1960).
[10] A. B. LERNER, T. B. FITZPATRICK, E. CALKINS and W. H. SUMMERSON, *J. Biol. Chem.* **191**, 799 (1951).

pattern of the broad bean enzyme is that the substituted catechols containing a free carboxyl group, such as protocatechuic acid, caffeic acid and its ester, chlorogenic acid (3-*O*-caffeoylquinic acid) are particularly poor substrates when judged on a rate basis. A similar observation has been made with regard to the substrate specificity of blowfly ortho-diphenol oxidase.[11] Contrasting behaviour is exhibited by potato tyrosinase which oxidizes chlorogenic acid almost as rapidly as any substrate tested, an observation which has led to the proposal that the potato enzyme should be termed chlorogenic acid oxidase.[12] In contrast to mushroom tyrosinase which oxidizes protocatechuic acid at one-fifteenth the rate obtained with DOPA[13] the difference with the broad bean enzyme is almost 500-fold.

Although DOPA is similar in chemical structure to the other acids listed in Table 1 the presence of the electrophilic amino group in the vicinity of its carboxyl group is reflected in the increased acidity of the latter (pK_1 for the amino acid is 2·3 and for the other acids, 4·2–4·4[14]). Thus it seemed that the poorer activity shown by the weaker acids was associated with the carboxyl group and its state of the ionization. Accordingly the activities of two acids (caffeic and gallic) both in the free state and as esters were determined at pH 6·8 and compared to the values at pH 5·0 (Table 2). The results show that methylation of the carboxyl

TABLE 2. THE OXIDATION OF ORTHODIPHENOLIC CARBOXYLIC ACIDS AND ESTERS AT pH 5·0 AND 6·7

Substrate	Relative rate*		% Change in rate at pH 6·5–6·8 compared to pH 4·8–5·2
	pH 4·8–5·2	pH 6·5–6·8	
DOPA	100	100†	−9
Protocatechuic acid	0·22	2·5	+900
Caffeic acid	33	46	+31
Methyl caffeate	48	62	+25
Chlorogenic acid	16	13	−15
Gallic acid	0·43	0·33	−30
Methyl gallate	2·9	4·0	+37

* Results (relative to DOPA equals 100) were obtained with the manometric assay using an α_2 fraction (purified 2100-fold) and 4 mg substrate. Caffeic and gallic acids and their esters were dissolved in an aqueous dioxane solution and hence were assayed in 1% dioxane, a concentration which had no discernible effect on enzyme action.

† Rate relative to pH 4·8–5·2 is 91; the figures in column 4 are corrected for this effect.

groups although leading to an increase in the rate does not render the substrate as liable to oxidation as the corresponding phenol (catechol and pyrogallol) at either pH value. Another significant property relates to the relatively large increase in the rate at which protocatechuic acid is oxidized at more neutral pH values, in contrast to the decrease in rate observed with simpler phenols. However protocatechuic acid appears to be an exception for little change in rate is noted for gallic, caffeic and chlorogenic acids.*

* Preliminary work indicates that *p*-coumaric acid is oxidized more rapidly at pH 6·8 than at pH 5·0.

[11] P. KARLSON and H. LIEBAU, *Z. Physiol. Chem.* **326**, 135 (1961).

[12] F. A. M. ALBERGHINA, *Phytochem.* **3**, 65 (1963).

[13] I. Z. EIGER and C. R. DAWSON, *Arch. Biochem. Biophys.* **21**, 194 (1949).

[14] G. KORTUM, W. VOGEL and K. ANDRUSSOW, *Dissociation Constants of Organic Acids in Aqueous Solutions*. Butterworths, London (1961).

Determination of the Michaelis Constant

In order to obtain a measure of the affinity of broad bean tyrosinase for several substrates the Michaelis constant was evaluated according to the method of Lineweaver and Burk from data obtained with several assay methods. A derivation made from the results tabulated in Table 3 is that the enzyme appears to have a lower affinity for DOPA, which is the major phenolic component found in bean tissues. Very similar values for the Michaelis constant have been reported for other related plant oxidases such as the tyrosinase of potato[12, 15] and various ortho-diphenol oxidases†[16–18] but they are distinct from *Neurospora*, mushroom, and mammalian tyrosinases where the Michaelis constant for DOPA is in the range of 0·2–0·8 mM.[19–23] With catechol as the substrate, values in the range of 4·0 to 0·17 mM have been reported for the mushroom enzymes.[3, 24]

TABLE 3. THE MICHAELIS CONSTANT OF BROAD BEAN TYROSINASE[a] FOR VARIOUS SUBSTRATES AND INHIBITORS AT pH 5·0

Substrate	Inhibitor	Class	Michaelis constant, K_m or K_i (mM)	Assay system[b]
Homocatechol	Nil		1·4, 4·0	Ferricyanide
			1·1	Ascorbic acid
			2·1, 4·3	Oxygen cell
Pyrogallol			2·3	Oxygen cell
DOPA			13, 14	Oxygen cell
			15	Ferricyanide
			12*	Manometric
Caffeic acid			1·0	Oxygen cell
p-Cresol			1·3*, 1·8*, 3·2*	Manometric
Homocatechol	Protocatechuic acid	Competitive	1·7	Ferricyanide
	Phenol	Competitive	18	
	4-Chlorophenol	Non-competitive	0·63	
	4-Nitrophenol	Non-competitive	0·79	
	Resorcinol	Non-competitive	0·12	
	3,4,5-Tri-methylphenol	Mixed	0·09	
p-Cresol	Sodium fluoride	Non-competitive	13*	Manometric
	Protocatechuic acid	Competitive	3·4	
DOPA	Sodium fluoride	Non-competitive	120*	Manometric

[a] The data relates to an enzyme purified 150-fold except where asterisked when an α_2 component purified 2100-fold was used.

[b] Ferricyanide and ascorbic acid refer to the spectrophotometric assays described in the Experimental section.

† The collective term "ortho-diphenol oxidases" refers to a closely related group of enzymes which have similar substrate specificities to tyrosinases with the important exception that they have no action on monophenols.

[15] C. WARNER, *Australian J. Res.* **B4**, 554 (1951).
[16] O. BJÖRKMAN and P. HOLMGREN, *Physiol. Plantarum* **13**, 582 (1960).
[17] A. M. MAYER, *Physiol. Plantarum* **14**, 322 (1961).
[18] J. K. PALMER, *Plant Physiol.* **38**, 508 (1963).
[19] A. S. SUSSMAN, *Arch. Biochem. Biophys.* **95**, 407 (1961).
[20] N. H. HOROWITZ and S.-C. SHEN, *J. Biol. Chem.* **197**, 513 (1952).
[21] K. YASUNOBO, In *Pigment Cell Biology* (Edited by M. GORDON). Academic Press, New York (1959).
[22] S. OSAKI, *Arch. Biochem. Biophys.* **100**, 378 (1963).
[23] K. SHIMAO, *Biochem. et Biophys. Acta* **62**, 205 (1962).
[24] M. A. EL-BAYOUMI and E. FRIEDEN, *J. Am. Chem. Soc.* **79**, 4854 (1957).

Inhibition of the Broad Bean Tyrosinase

In common with other tyrosinases[25] excessive amounts of a substrate inhibit the broad bean enzyme. For example at a concentration of 260 mM homocatechol the enzyme is inhibited to the extent of 90 per cent when compared with the optimal concentration of 11 mM. Therefore, to avoid such inhibition in the experiments reported below, sub-optimal concentrations of substrate (viz. homocatechol, DOPA and *p*-cresol) were employed.

The inhibitors studied comprised various phenols and sodium fluoride; the degree of inhibition at pH 5·0 was determined at several substrate concentrations using either the manometric method or the spectrophotometric assay employing ferrocyanide. The results obtained were analysed by three graphical methods where the ordinates are $1/v$, v and s/v and the corresponding abcissae are $1/s$, v/s and s respectively and an example of one plot is reproduced in Fig. 1. Under the chosen conditions only phenol and protocatechuic acid were identified as true competitive inhibitors of homocatecholase activity (see Table 3).

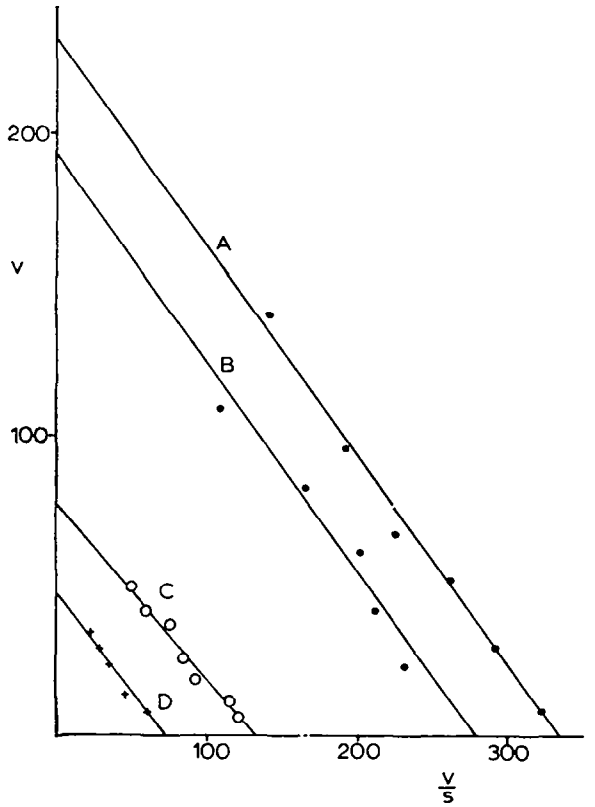

FIG. 1. PLOT OF INITIAL VELOCITY, DETERMINED BY FERROCYANIDE ASSAY, FOR HOMOCATECHOL ALONE (CURVES A AND B) AND IN THE PRESENCE OF 1·2 MM *p*-NITROPHENOL AND 1·3 MM *p*-CHLOROPHENOL (CURVES C AND D RESPECTIVELY).

Compounds showing non-competitive inhibition were 4-nitrophenol, 4-chlorophenol, resorcinol and sodium fluoride, whilst 3,4,5-trimethylphenol was shown to be a mixed type inhibitor which affects the affinity of the enzyme for its substrate (the modified Michaelis constant for homocatechol being 7·1 mM) and yet combines at another site.[26]

[25] W. H. MILLER and C. R. DAWSON, *J. Am. Chem. Soc.* **64**, 2344 (1942).

[26] M. DIXON and E. C. WEBB, *Enzymes* (1st Ed.). Longmans, Green, London (1957).

4-Nitrophenol, resorcinol and 4-chlororesorcinol have previously been classified as competitive inhibitors[27–29] of tyrosinases and indeed the conception that they should bind with the normal substrate binding site, to some extent, is very credible. Richter[28] concluded that resorcinol acted competitively on the basis that inhibition was reduced when excess substrate was added. When a similar experiment was performed with *p*-nitrophenol and the broad bean enzyme an identical result was obtained and thus it is possible that at higher substrate concentrations the phenols characterized above as non-competitive inhibitors would assume competitive behaviour.

The Oxygen Dependence of Broad Bean Tyrosinase

The oxygen dependence of the enzyme was investigated using the manometric method by determining the Michaelis constant for oxygen at various levels of *p*-cresol. When the Lineweaver–Burk plots were constructed a series of approximately parallel lines resulted showing that the affinity of the enzyme for oxygen is dependent on the *p*-cresol concentration. This effect is illustrated in Table 4. The Michaelis constant for *p*-cresol as determined from a

TABLE 4. INTERDEPENDENCE OF THE MICHAELIS CONSTANTS FOR OXYGEN AND *p*-CRESOL WITH THE CONCENTRATION OF THE SECOND SUBSTRATE

p-Cresol concentration (mM)	K_m for oxygen (μM)	Oxygen tension (%)	K_m for *p*-cresol (mM)
0·62	24·5	1·72	1·2
1·54	30·0	4·54	1·5
3·08	46·5	16	2·3
7·70	58·5	21	2·7
15·4	66·0	100	1·5

secondary plot of the intercepts and reciprocal of the corresponding *p*-cresol concentration gave a value of 1·6 mM, in good agreement with that reported previously. The complementary experiments where the affinity of the enzyme for DOPA and *p*-cresol with varying oxygen tensions was determined, gave Lineweaver–Burk plots similar in form to those for oxygen except that in this case (*a*) the intercept is linearly related to the oxygen tension applied and thus the Michaelis constant is also directly related to the concentration of oxygen (cf. Table 4) and (*b*) the slopes were only parallel with *p*-cresol, and not with DOPA.

DISCUSSION

In a previous communication[1] the theme was developed that the configurational properties of the broad bean tyrosinase molecule are more reminiscent of those possessed by the enzyme isolated from *Neurospora crassa* than that from *Agarious bispora*. With regard to substrate specificity however, the broad bean enzyme is distinct from both and the affinity it has for substrates is more comparable with the "phenol oxidase" from higher plants, potato (a tyrosinase),[12, 15] lettuce[17] and *Solidago*[16] (both *o*-diphenol oxidases). Such variations in substrate specificity are presumably reflected in different configurations at the active centres of the different enzymes.

[27] J. BONNER and S. G. WILDMAN, *Arch. Biochem.* **10**, 497 (1946).
[28] D. RICHTER, *Biochem. J.* **28**, 901 (1934).
[29] H. HEYMANN, Z. ROGACH and R. L. MAYER, *J. Am. Chem. Soc.* **76**, 6330 (1954).

However it is expected that many properties of the active centre are common to all tyrosinases and in this context inhibition by acid may be quoted. Thus Krueger[30] and Keilin[31] reported that both sodium halides and azide inhibited mushroom tyrosinase—observations which may be extended to the mammalian,[32] broad bean, potato and apple enzymes. Kuttner and Wagreich[33] and Krueger[30] extended the earlier work of Ludwig and Nelson[34] and demonstrated that aromatic carboxylic acids, especially benzoic acid, and other non-aromatic acids, notably oxalic acid, were also inhibitors of mushroom tyrosinase. The kinetics of inhibition appeared to be different for the inorganic salts and benzoic and oxalic acids respectively. Krueger identified only oxalic acid as acting competitively but an earlier report[15] classified 3-hydroxybenzoic acid as a competitive inhibitor, a conclusion which has more recently been extended to benzoic acid also.[21]

A characteristic common to all these agents is that their effectiveness is markedly affected by pH changes, being greater at low pH. Both Krueger[30] and Yasunobo[21] have suggested that these inhibitors combine at a positive site on the enzyme molecule and since the inflection point of the pH dependence occurs at a pH of approximately 6 they conclude that a histidine moiety which must also be regarded as part of the active centre, may be involved. According to this scheme therefore, the anionic species of each inhibitor is the active agent.

However, the fact that 3,4-dihydroxybenzoic acid (protocatechuic acid) is a poor substrate and also is more rapidly oxidized at pH 7 than pH 5 is difficult to reconcile to this theory. If protocatechuic acid behaves in an analogous manner to 3-hydroxybenzoic acid the enzyme would be expected to possess a higher affinity for it at the lower pH and concomitantly a greater rate of oxidation would be predicted.

In a study of the pH dependence of the oxidation of protocatechuic acid, made with a mushroom tyrosinase preparation (see below for experimental details), it was found that the enzyme did indeed possess a smaller K_m at pH 5·1 than at pH 7·2 (the values being 0·17 mM and 3·3 mM respectively). The maximal velocity however was five times greater at pH 7·2 than pH 5·1. Such a change in the velocity function might be expected to be associated with the decrease in the relatively high redox potential of protocatechuic acid (E_0 0·883 V) as the pH is increased. The fact that the K_m for protocatechuic acid does vary in a pH range where the K_m for butylcatechol is virtually constant (see Table 5) is difficult to interpret but presumably is due to either an ionization in the protein as suggested earlier,[32] or to an ionization in the protocatechuic acid. It is the later idea which is more fully developed in this paper.

Hence the alternative hypothesis is proposed that the undissociated acid is responsible for inhibition and that copper present at the active centre is implicated in the inhibitory process. Support for the first part of this statement has been derived from data obtained with a purified mushroom tyrosinase. The inhibition caused by fluoride, azide and 4-nitrocatechol was studied over the pH range 4·1–8·0 using the oxygen cell assay modified by the omission of manoxal, and the substitution of 4-*t*-butylcatechol as substrate. The variation of the Michaelis constants and, the inhibitor constants with pH is illustrated in Table 5. The dissociation constants (K_i) for the enzyme–inhibitor complexes appear to decrease as the pH is lowered and for fluoride this observation applies even in a region where the Michaelis constant increases. However, when the inhibitor constants are corrected on the assumption

[30] R. C. KRUEGER, *Arch. Biochem. Biophys.* **76**, 87 (1958).
[31] D. KEILIN, *Proc. Roy. Soc.* (*London*) **B121**, 165 (1937).
[32] A. B. LERNER, *Arch. Biochem. Biophys.* **36**, 473 (1952).
[33] R. KUTTNER and H. WAGREICH, *Arch. Biochem. Biophys.* **43**, 80 (1953).
[34] B. J. LUDWIG and J. M. NELSON, *J. Am. Chem. Soc.* **61**, 2601 (1939).

that only the undissociated acids are giving rise to the inhibition the inhibitor constants no longer show a significant pH dependence and thus it is inferred that the pH affects the inhibitor rather than the inhibitor binding site on the enzyme.

TABLE 5. INHIBITION OF MUSHROOM TYROSINASE WITH FLUORIDE, AZIDE AND 4-NITROCATECHOL

(a) The variation with pH of the dissociation constant of the fluoride, azide and 4-nitrocatechol enzyme complexes. See text for experimental details.

System	pH	Apparent K_m and K_i (mM) 4·1 (mM)	5·2 (mM)	6·1 (mM)	7·0 (mM)	8·0 (mM)	Type of inhibition
Michaelis constant with *t*-butylcatechol		8·0	1·5	2·6	1·6	2·0	
Inhibition by fluoride		0·25	1·4	12·2	45·0	—	Non-competitive
Inhibition by azide		0·02	0·02	0·17	1·76	—	Mixed
Inhibition by 4-nitrocatechol		—	—	0·18	0·32	1·36	Competitive

(b) pK_i calculated on the assumption that the *un*dissociated acid only is responsible for inhibition.*

Inhibitor†	pH	pK_i 4·1	5·2	6·1	7·0	8·0
Hydrofluoric acid		4·35	4·60	4·55	4·90	—
Hydrazoic acid		4·80	5·25	5·15	5·05	—
4-Nitrocatechol		—	—	3·85	3·90	4·10

* This correction has been made by applying the formula $\log(1/K_i) + \log K_{A/H^+} + 1$ where K_A is the dissociation constant for the acid inhibitor studied. The K_A for hydrofluoric[36] and hydrazoic acids[37] has been taken as $3{\cdot}53 \times 10^{-4}$ and $1{\cdot}9 \times 10^{-5}$ respectively; that for 4-nitrocatechol was estimated by titration to be about $1{\cdot}6 \times 10^{-7}$.

† The normalized pK_i for the broad bean tyrosinase–hydrofluoric acid complex at pH 5·0 is 3·45 and 2·5 with *p*-cresol and DOPA as substrates respectively.

Table 5 also shows that the mushroom enzyme has a much higher affinity for hydrofluoric acid than the broad bean. A similar variation has also been reported by Warner[15] who reported that equivalent concentrations of 3-hydroxybenzoic acid inhibited the mushroom enzyme more strongly than the potato tyrosinase.

Fluoride, azide and benzoic acids are known to chelate copper *in vitro* and it seems reasonable to presume that the copper of tyrosinase is likewise affected. In this context tyrosinase may be compared to caeruloplasmin, catalase and peroxidase where a strongly pH dependent inhibition, which is more intense at low pH, is also produced by "anions".[35,38,39] Thus Agner and Theorell[35] concluded that any anion in high enough concentration inhibited catalase and Chance[37] elucidated the mechanism in the terms that the undissociated acid displaced a water molecule attached to the metal at the active centre. Thus it is suggested that in pH regions near the pK of the inhibitor (where the inhibitor is most effective), the inhibition produced is due to the formation of a complex between copper at the active centre and the

[35] K. AGNER and H. THEORELL, *Arch. Biochem.* **10**, 321 (1946).
[36] C. D. HODGMAN (Ed.), *Handbook of Chemistry and Physics* (44th Ed.). Chemical Rubber, Cleveland, Ohio (1963).
[37] International Critical Tables.
[38] C. G. HOLMBERG and C.-B. LAURELL, *Acta Chem. Scand.* **5**, 921 (1951).
[39] B. CHANCE, *J. Biol. Chem.* **194**. 483 (1952).

undissociated acid. The possibility that the anion also complexes with the functional copper cannot be excluded, although the stability of such a complex is much lower.

An explanation for the fact that DOPA is a much better substrate than protocatechuic and gallic acids can also be based on the assumption introduced above. For an acid which is also a substrate, the possibility arises that two enzyme complexes may be formed—namely the enzymically active complex and an inactive one in which the enzymic copper is linked through the undissociated carboxyl group. In the pH range 5–7 the carboxyl group of DOPA is almost wholly ionized and thus such an inactive complex is unlikely to be produced. In contrast protocatechuic acid is ionized to the extent of 80 per cent at pH 5·0 and the formation of an inactive enzyme complex is more favoured. Hence the pH dependence of the rate of oxidation of this acid can be explained on the basis that as the pH is increased the concentration of the active complex is increased and an enhanced rate of oxygen uptake is observed. It was noted earlier that the behaviour of protocatechuic acid is exceptional and application of this argument to other substrates must be coupled with a consideration of steric factors. Any substitution of benzoic acid reduces its inhibitory efficiency[30, 33] and this fact favours the conclusion that in the other weakly acid substrates, opportunity for chelation through the carboxyl group is reduced and thus reactivity is higher and not dependent on pH to the same extent. It has also been demonstrated[30] that benzamide and ethylbenzoate when compared to benzoic acid show much reduced potency as inhibitors. This fact is reflected in the increased reactivity of the methyl esters tested but the pH dependence which they also show remains unexplained.

No attempt at a full analysis of the effect of oxygen on the cresolase and diphenolase (catecholase) activities of tyrosinase can be offered. To summarize the results using Dalziel's nomenclature,[40] it can be seen that when *p*-cresol is oxidized ϕ_{12} approximates to zero, since curves of constant slopes are obtained. On the other hand, when DOPA is oxidized, the slopes are not constant and the intercept is directly proportional to the reciprocal of the oxygen tension (i.e. ϕ_0 approximates to zero).

Many mechanisms consistent with the kinetics of DOPA oxidation can be formulated. One example is the TYPE III mechanism of Mason.[41] Expressed in its simplest form it is

$$E + O_2 \rightleftharpoons EO_2$$

$$EO_2 + DH_2 \rightarrow EO + D + H_2O$$

$$EO + DH_2(\text{or AH}) \rightarrow E + D + H_2O(\text{or } E + DH_2)$$

Where E, O_2, DH_2 and AH represent enzyme, oxygen, *o*-dihydroxyphenol and monophenol respectively. This mechanism predicts that ϕ_{12} is zero when *p*-cresol is the substrate but there is a disagreement with the data which show that the intercepts of a plot of $1/v$ against $1/s$ with this substrate are not linearly related to the reciprocal of the oxygen tension.

EXPERIMENTAL

Chemicals

Homocatechol was purified by recrystallization to constant m.p. from petroleum ether, *t*-butylcatechol by sublimation and *p*-cresol by redistillation twice under reduced pressure. Other chemicals were best grade commercial products used without further purification.

[40] K. Dalziel, *Acta Chem. Scand.* **11**, 1706 (1957).
[41] H. S. Mason, *Advances Enzymol.* **19**, 79 (1957).

Preparation of the Enzyme

a. From broad bean. The detailed purification of broad bean tyrosinase has been outlined elsewhere.[1] Briefly, the method involves extraction of a lyophilized or acetone powder of crude leaf protein, followed by fractionation with ammonium sulphate, triethylaminoethyl-cellulose (TEAE) and hydroxylapatite. Three enzyme preparations of different degrees of purity were employed in this study. One preparation, purified 150-fold, was obtained after fractionation on TEAE-cellulose. A second was further purified to over 2000-fold, and the third was a fraction purified 500-fold.

b. From mushroom. After application of steps 1–6 as outlined by Bouchilloux *et al.*[4] the preparation was adsorbed onto a DEAE-Sephadex column equilibrated with 0·02 M phosphate buffer, pH 7·0 and the enzyme was recovered in 70 per cent yield using a linear gradient developed with 0·1 M phosphate, 0·05 M citrate buffer pH 5·2. The specific activity of this preparation was 2750 chronometric units/mg protein[42] which represents a purification of 200-fold over the extract obtained from Step 1.

Enzyme Assay

1. Two methods were employed to measure the initial linear rate of oxygen consumption using either a monohydroxy- or an orthodihydroxyphenol as substrate. (*a*) In the manometric assay a total volume of 3·0 ml was used: activated enzyme (i.e. a mixture of enzyme and 1 μmole manoxal at pH 5·0 was added from the sidearm to a solution of the substrate in either 0·1 M phosphate, 0·05 M citrate buffer pH 5·0, or 0·1 M phosphate buffer pH 6·8. The reaction was carried out at $25 \pm 0{\cdot}05°$ with a shaking rate of 105/min and, potassium hydroxide was used in the centre well only when substrates containing a carboxyl group were assayed. For the data given in Table 4 a trace of homocatechol (0·01 mg) was also added in order to diminish the lag period which precedes the oxidation of monophenols. (*b*) The second method utilized an oxygen cell made according to the general design of Clarke.[43] For determinations made at pH 5·0 the cell was immersed in 3·0 ml of a solution of enzyme in 0·1 M phosphate–0·05 M citrate buffer at pH 5·0, maintained at $25 \pm 0{\cdot}05°$ and the mixture was stirred magnetically. Manoxal (1 μmole), was added 1 min before the substrate to give a final volume of 3·5 ml. The disappearance of oxygen was measured as a drop in potential on a recorder and the rate of the initial linear drop was used as a measure of enzyme activity. Since the activating effect of manoxal is much more pronounced at pH 5·0 than at pH 6·8, this procedure was modified when determinations were made at the higher pH value. The enzyme and manoxal were added to buffer at pH 5 (2·0 ml) and 1 min later 0·1 M trisodium phosphate buffer (pH 10·0) was added to give a final pH of 6·8. After the oxygen cell had been immersed in the solution, the substrate was then added to give a final volume of 3·5 ml.

2. At pH 5·0 the redox potentials of the orthoquinones produced from substrates are generally sufficiently high to bring about the rapid oxidation of both potassium ferrocyanide and ascorbic acid. Sussman[19] and El Bayoumi and Frieden[24] have shown that tyrosinase activity toward several substrates can be measured spectrophotometrically by following the oxidation of these compounds and these methods have been adopted in this study.

With two exceptions consistent results were obtained with these various systems. Differences were observed when (*a*) the spectrophotometric method based on the oxidation of

[42] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).
[43] L. C. CLARKE, Jr., *Trans. Am. Soc. Artificial Internal Organs* **2**, 41 (1956).

ferrocyanide was used with pyrogallol as a substrate, here the activity relative to homocatechol was lower (by a factor of approximately three) when compared with the methods based on oxygen consumption and is perhaps due to the inadequate rate of reaction between ferrocyanide and the 3-hydroxy-*o*-benzoquinone: (*b*) the activity of *p*-cresol relative to homocatechol was lower (also by a factor of approximately three) when estimated using the oxygen cell compared with the manometric assay.

Acknowledgements—This work was supported largely by a grant administered under PL 480 and subsequently by one from the U.S. Public Health Service.