[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

The Oxidation of Catechol-type Substrates by Tyrosinase

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The fundamental function of the enzyme tyrosinase is thought to be the catalysis of the oxidation of the *ortho*-dihydroxy group of a catechol to an *ortho*-quinone. The enzyme is primed during this oxidation and after this priming is able to bring about the oxidation of monohydric phenols.[2] Robinson and McCance[3] first showed that the enzymatic oxidation of catechol requires two atoms of oxygen per mole. Since the production of an *ortho*-quinone from catechol requires only one atom of oxygen some explanation had to be made as to how the second atom was consumed. Onslow and Robinson[4] suggested that the second atom of oxygen resulted in the formation of hydrogen peroxide

$$C_6H_4(OH)_2 + O_2 \longrightarrow C_6H_4O_2 + H_2O_2$$

Several workers have expressed doubt concerning the validity of this postulate. The arguments advanced by these workers have been summarized in a paper by Dawson and Ludwig[5] who have presented additional evidence against the hydrogen peroxide postulate.

According to Dawson and Nelson[6] the oxidation of catechol takes place in two steps. Only the first step, the oxidation of catechol to *o*-benzoquinone, is catalyzed by the enzyme. This step consumes one atom of oxygen. The quinone reacts with water to form hydroxyhydroquinone, which in turn becomes oxidized to an *o*-quinone, thus consuming the second of oxygen. Later doubt was cast on this mechanism by Mason, Schwartz and Peterson[7] whose examination of the absorption spectrum of catechol undergoing oxidation in the presence of tyrosinase failed to detect hydroxyhydroquinone.

Since the oxidation of a monohydric phenol is through conversion to a catechol-type with the consumption of one atom of oxygen and subsequent oxidation of the catechol with the consumption of two atoms of oxygen, it follows that the oxidation of phenol requires three atoms of oxygen per mole.

Considerable data are recorded in the literature on the extent of oxidation of various phenolic substances but they are scattered and without attempted correlation. This is a natural result of their bit by bit presentation wherein little attention has been given to the effect of structure on the extent of oxidation. It would be an advantage to the advance of the knowledge of enzymatic oxidations if more were understood about the role variation of structure plays in extent and rate of oxidation.

This paper is a report of a study of the effect of variation in structure of the substrate on the extent of its oxidation. In approaching this problem the following postulates were used as guides:

1. It appears to be a general rule that addition to an ortho-quinone results principally in the formation of a 4-substituted catechol.
2. Dawson and Nelson[6] have suggested that water adds in the same way.
3. The use of a second atom of oxygen in the enzymatic oxidation of catechol depends upon the addition of water to *o*-benzoquinone.
4. It seemed likely that if substituents were placed in the 4-position of catechol this might prevent the hydration of the *o*-quinone and thus limit the oxygen consumption to one atom per mole.

Several catechol-type substrates were used to test the above postulates. These were allowed to oxidize in the presence of the enzyme until no more oxygen was consumed. The results are tabulated and discussed in the following section.

The Effect of Substituent Groups on the Total Oxygen Consumption of Catechol-Type Substances.—In Table I there is recorded the result of the oxidation to completion of several catechol-type substances when in the presence of tyrosinase. The oxygen consumption was measured by the technique of the Warburg respirometer, according to Graubard and Nelson.[8]

When the above results are considered in the light of the nature of the groups which are substituents the following generalizations seem permissible:

1. An *ortho*-directing group in the 4-position does not alter the amount of oxygen from that required by catechol.
2. A *meta*-directing group brings about a change in the oxygen consumption, only one atom of oxygen being used in place of the two of catechol.
3. When both the 4- and 5-positions are substituted by *ortho*-directing groups the oxygen consumption is only one atom per mole of substrate.

This would seem to indicate that the 4-position is of great importance in the consumption of the

(1) An extract of the thesis submitted by the author to the faculty of Columbia University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

(2) Chas. A. Bordner and J. M. Nelson, THIS JOURNAL, **61**, 1507 (1939).

(3) M. E. Robinson and R. A. McCance, *Biochem. J.*, **19**, 251 (1925).

(4) M. W. Onslow and M. E. Robinson, *ibid.*, **20**, 1138 (1926).

(5) C. R. Dawson and B. J. Ludwig, THIS JOURNAL, **60**, 1617 (1938).

(6) C. R. Dawson and J. M. Nelson, "Advances in Enzymology," IV, 99 (1944).

(7) Mason, Schwartz and Peterson, THIS JOURNAL, **67**, 1233 (1945).

(8) Mark Graubard and J. M. Nelson, *J. Biol. Chem.*, **111**, 757 (1935).

TABLE I

OXYGEN CONSUMPTION OF CATECHOL-TYPE SUBSTRATES DURING OXIDATION CATALYZED BY TYROSINASE

Group	Position	Atoms of oxygen per mole substrate: Author	Others	Reference
—Cl	4	2.1		
—CH_3	4	...	2.0	(9)
—CHO	4	1.1		
—COOH	4	1.1		(10)
—$COCH_3$	4	1.0		
—$COCH_2Cl$	4	1.1		
—SO_3Na	4	1.0		
—NO_2	4	0.0		
—$SO_2C_6H_5$	4	...	0.0	(9)
—$COCH_2NHCH_3$	4	...	0.0	(11)
—SO_3Na	3	0.0		
—NO_2	3	1.9		
—CH_3	4, 5	1.0		
—Cl	4, 5	1.1		
—Br	4, 5	1.0		
—NO_2	3, 5	0.0		
—Cl	3, 5	1.0		
—Br	3, 5	1.0		
—Cl	3, 4, 5	1.0		
—Br	3, 4, 5, 6	1.1		
—Br, (—CH_3)	3, 5, 6 (4)	1.0		

Measurements were made at 25°. The reaction flasks were of 50 ml. volume and contained as the reaction mixture 1 ml. of enzyme added from the side-arm at zero time, 2 ml. of (0.2 *M*) phosphate–(0.1 *M*) citrate buffer to produce a *p*H of about 7, substrate solution, and water to bring the volume to 8 ml. The enzyme used was purified extract from the common mushroom, *Psalliota campestris*. When the absorption of oxygen ceased fresh enzyme was added. This was repeated until addition of enzyme failed to cause the absorption of more oxygen. The amount of substrate used was 1.0×10^{-5} mole, except in a few cases where it was less due to the low solubility of the substrate. The results are the average of three or more determinations.

second atom of oxygen by catechol. In those substances where the 4-position is occupied by a substituent, flanked by a *meta*-directing group, or by two *ortho*-directing groups the consumption of the second atom of oxygen does not occur. The result in all cases is the production of an *o*-quinone, without formation of hydrogen peroxide.

The Effect of Substituent Groups on the Total Oxygen Consumption of Monohydric Phenols.—Since the introduction of substituent groups onto the catechol ring has a marked effect on the extent of oxidation of catechols it was considered of interest to examine the effect of these substituents on the ability of the enzyme to introduce a second hydroxyl group into monohydric phenols.

Data from the literature on the extent of oxidation of monohydric phenols have been used in compiling Table II which classifies the phenols according to the nature and position of the substituent. Only two new substances have been studied here.

(9) C. E. Pugh and H. S. Raper, *Biochem. J.*, **21**, 1370 (1927).

(10) R. D. H. Heard and H. S. Raper, *ibid.*, **27**, 36 (1933).

(11) D. Baker and J. M. Nelson, *J. Gen. Physiol.*, **26**, 269 (1943).

TABLE II

OXYGEN CONSUMPTION OF SUBSTITUTED PHENOL DURING OXIDATION CATALYZED BY TYROSINASE

Group in position: 2	3	4	5	Atoms of O_2 per mole	Reference
CH_3	....	.....	..	0	9
CH_3	....	CH_3	..	0	12
CH_3	....	.....	CH_3	0	12
CH_3	CH_3	.....	CH_3	0	12
OCH_3	....	.....	..	0	9, 12
CHO	....	.....	..	0	9
COOH	....	.....	..	0	9
NO_2	....	.....	..	0	9
....	CH_3	.....	..	3	9,12
....	OCH_3	.....	..	3	9
....	Cl	.....	..	0	12
....	C_2H_5	.....	..	2	12
....	CH_3	.....	CH_3	0	12
....	CH_3	CH_3	..	2 Author's obs.	12
....	C_2H_5	.....	CH_3	0	12
CH_3	CH_3	.....	..	2	12
....	COOH	.....	..	0	9
....	....	CH_3	..	3	9,12
....	....	OCH_3	..	3	9,12
....	....	*t*-C_4H_9	..	1	12
....	....	*n*-C_4H_9	..	3	12
....	....	Cl	..	3.0 Author's obs.	
....	....	CHO	..	0[a]	
....	....	COOH	..	0[a] Author's	13
....	....	$COCH_3$	..	0[a] Author's	
....	....				

[a] After eight hours contact with the enzyme no oxidation had taken place. Attempts to prime the enzyme with traces of added catechol failed to bring about oxidation.

By comparison of the results presented in this table with those of Table I it can be seen that the same groups which act to limit the oxidation of catechols to a one-atom-per-mole stage here operate to prevent the introduction of a new or second hydroxyl group by the enzyme. The resulting substituted phenol is inert to tyrosinase oxidation. This seems to be true no matter what is the position of the substituent group. It also may be noted that there are some monohydric phenols which are not subject to the oxidative influence of tyrosinase even when their catechol analogs are substrates.

The oxidation of catechol and a few members of the catechol family by tyrosinase has been long-since well demonstrated. However, if tyrosinase is to play an important part in cellular respiration, as has been recently postulated,[10,14,15] it should possess the ability to catalyze the oxidation of a multitude of diverse and complexly substituted catechols, whose occurrence is widespread. This can now more certainly be considered to be a real property of the enzyme in the light of the findings of this paper.

The enzyme must also possess the property of being primed toward monohydric phenols when it

(12) J. Enselme and J. L. Vigneau, *Bull. soc. chim. biol.*, **27**, 387 (1945).

(13) M. W. Onslow and M. E. Robinson, *Biochem. J.*, **19**, 420 (1925), stated that this gave a positive test for catechol after it had stood twenty-four hours with potato tyrosinase.

(14) E. S. Robinson and J. M. Nelson, *Arch. Biochem.*, **4**, 111 (1944).

(15) E. M. Walter and J. M. Nelson, *ibid.*, **6**, 131 (1945).

catalyzes the oxidation of the more complexly substituted catechols. Without this property the enzyme would be devoid of power to aid in the utilization of the monohydric phenols in nature. The question as to whether the oxidation of a given substrate causes the enzyme to be primed for the oxidation of monohydric phenols must be tested by the use of a substance whose oxidation by tyrosinase alone is negligible. While hydroquinone is not a monohydric phenol it behaves as such toward the enzyme. Its oxidation is very slow and one can note the priming effect of adding traces of catechol. Gregg and Nelson[16] have shown that the oxidation of catechol primed the enzyme so that it could bring about the oxidation of hydroquinone. We have tested the priming of the enzyme during the oxidation of several of the substrates reported in this paper. The results are shown in Table III.

TABLE III

SHOWING THE ABILITY OF TYROSINASE TO EFFECT THE OXIDATION OF HYDROQUINONE DURING OXIDATION OF CATECHOL-TYPE SUBSTRATES

Substrate concentration, 0.25 × 10^{-5} mole; *p*H 6.40; hydroquinone, 5 mg.; gelatin, 5 mg.; enzyme, 2.75 catecholase units[16]

Substrate	Oxygen (cubic millimeters) Required	Consumed (30 min.)
Catechol	56.0	420
3,4-Dihydroxybenzenesulfonate Na	28.0	400
3,5-Dichlorocatechol	28.0	375
3,4,5-Trichlorocatechol	28.0	290
3-Nitrocatechol	56.0	225
3,4-Dihydroxy-ω-chloroacetophenone	28.0	130

The oxygen uptake in excess of that calculated as required for the catechols was consumed in the oxidation of the added hydroquinone. Thus it is seen that regardless of whether the substrate consumes one or two atoms of oxygen it can cause the enzyme to be primed to oxidize monohydric phenols.

Discussion

The question of hydrogen peroxide formation during the oxidation of catechols and phenols has been marked by controversy since the postulate of its formation was first advanced. In recent years the weight of evidence has been such that this postulate is being for the most part rejected. The finding of several substrates whose oxygen consumption is limited to a single atom of oxygen per mole adds confirmation to the work of those who reject the hydrogen peroxide postulate. In all cases where one atom of oxygen was consumed the product was found to behave as an *o*-quinone, and no hydrogen peroxide could be detected. The enzyme being free from catalase, the conclusion that there is no hydrogen peroxide formed appears well justified.

(16) Donald C. Gregg and J. M. Nelson, THIS JOURNAL, **62**, 2500 (1940).

Studies on reaction rates of the system air–tyrosinase–catechol and air–tyrosinase–*p*-cresol are made difficult due to the complex nature of the reaction which each undergoes. The use of a substrate whose oxidation requires only one atom of oxygen per mole should eliminate many of these difficulties.

Experimental Details

Many of the catechol-type substrates used in this study were not available from commercial sources, thus necessitating their preparation according to the methods from previous literature as shown below.

4-Chlorocatechol.—By treating catechol with sulfuryl chloride; m. p. 90–92°.[17]

3,4-Dihydroxybenzaldehyde.—By treating piperonal with phosphorous oxychloride, followed by hydrolysis; yields very small; m. p. 152–153°.[18]

3,4-Dihydroxybenzoic Acid.—By fusion of vanillin with potash; m. p. 197–199°.[19]

3,4-Dihydroxyacetophenone.—By reduction of 4-chloroacetylcatechol; m. p. 116–117°.[20]

3,4-Dihydroxy-ω-chloroacetophenone.—By treatment of catechol with chloroacetic acid and phosphorous oxychloride.[21]

Sodium 3,4-Dihydroxybenzenesulfonate.—By treatment of guaiacol with sulfuric acid; m. p. 97–98°.[22]

4-Nitrocatechol.—By Dakin oxidation of 5-nitrosalicylaldehyde; m. p. 168–170°.[23]

3-Nitrocatechol.—By nitration of catechol; m. p. 86–90°.[24]

4,5-Dimethylcatechol.—From *o*-xylenol.[25]

4,5-Dichlorocatechol.—By treating catechol with sulfuryl chloride.[17]

4,5-Dibromocatechol.—By bromination of catechol in acetic acid.[26]

3,4,5-Trichlorocatechol.—By chlorination of catechol; m. p. 104–105°.[27]

Tetrabromocatechol.—Bromination of catechol; m. p. 192–193°.[28]

3,5,6-Tribromo-4-methylcatechol.—Bromination of 4-methylcatechol; m. p. 163–164°.[29]

3,5-Dibromocatechol.—By Dakin oxidation of 3,5-dibromosalicylaldehyde; m. p. 92–93°.[23]

3,5-Dichlorocatechol.—By Dakin oxidation of 3,5-dichlorosalicylaldehyde; m. p. 85–86°.[23]

3,5-Dinitrocatechol.—By nitration of catechol diacetate; m. p. 160–164°.[30]

Summary

1. It has been shown that the introduction of the *ortho*-directing groups —CH_3 and —Cl into the 4-position of the catechol ring produces a tyrosinase substrate whose oxygen consumption is the same as that for catechol, that is, two atoms of oxygen per mole.

2. The introduction into the 4-position of the catechol ring of such *meta*-directing groups as —CHO, —COOH, —$COCH_3$, —$COCH_2Cl$ and

(17) *Ber.*, **44**, 2175 (1911).
(18) *Monatsh.*, **14**, 382 (1883).
(19) *Ber.*, **7**, 617 (1874).
(20) *J. Chem. Soc.*, **105**, 1051 (1914).
(21) THIS JOURNAL, **57**, 1382 (1935).
(22) *Ber.*, **39**, 4093 (1906).
(23) *Am. Chem. J.*, **42**, 492 (1910).
(24) *Bull. soc. chim.*, [3] **9**, 53, 137 (1898).
(25) *Ber.*, **42**, 2922 (1909).
(26) *Ann. chim. phys.*, [7] **13**, 487 (1902).
(27) *Bull. soc. chim.*, [3] **13**, 719 (1902).
(28) *Am. Chem. J.*, **26**, 31 (1894).
(29) *Bull. soc. chim.*, **11**, 735 (1900).
(30) *Ber.*, **26**, 2183 (1893).

—SO_3Na produces substrates whose oxygen consumption is one atom per mole.

3. For those substrates in which both the 4- and 5- positions are substituted the oxygen consumption is one atom per mole.

4. It was shown that the —NO_2 group in the 3-position of the catechol ring produces a substrate which consumes two atoms of oxygen per mole, whereas the —SO_3Na group in this position produces a substance inert to tyrosinase.

5. Leaving the 4-position open and substituting in the 3- and 5-positions produces a substrate which consumes one atom of oxygen per mole.

6. Analysis of the literature shows that the oxidation of a monohydric phenol by tyrosinase fails when there is in the 2-position any group other than —OH.

7. Analysis of the literature shows that an *ortho*-directing group (except —OH) in the 3-position of phenol produces a substrate which consumes three atoms of oxygen, whereas if the group is *meta*-directing the substance is inert to tyrosinase.

8. When there is in the 4-position of the phenol ring an *ortho*-directing group the substance consumes three atoms of oxygen per mole. This was demonstrated with *p*-chlorophenol. When the group is *meta*-directing the substance is inert toward tyrosinase.

9. 4,5-Dimethylphenol was oxidized by tyrosinase with the consumption of two atoms of oxygen per mole.

10. Oxidation of catechol-type substrates enabled the enzyme to bring about the oxidation of hydroquinone.

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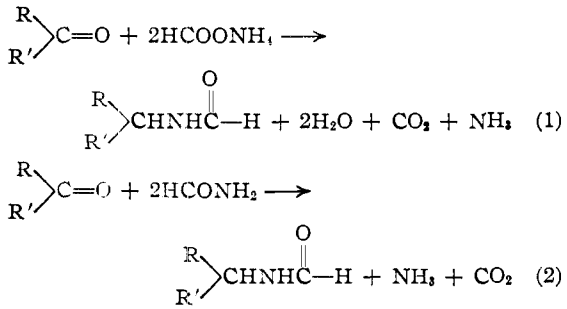
[CONTRIBUTION FROM THE NOYES CHEMICAL LABORATORY, UNIVERSITY OF ILLINOIS]

Studies on the Mechanism of the Leuckart Reaction

BY ELLIOT R. ALEXANDER AND RUTH BOWMAN WILDMAN

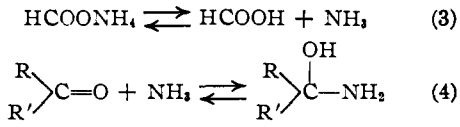
The conversion of carbonyl compounds into the corresponding amino derivatives by means of excess ammonium formate or formamide is usually called the Leuckart reaction. Recently this reaction has been used for the preparation of a rather large number of amines,[1] and the experimental conditions have been developed to the point where good yields can be obtained.

The mechanism of this reaction, however, is not definitely known. Superficially at least the equation may be written as in (1) or (2) in which either ammonium formate or formamide can be considered to be the essential reactant. It is quite interesting that both reagents have been used successfully, although ammonium formate appears to give somewhat better yields than formamide alone[1c,1d] or formamide in the presence of formic acid.[1b] These facts suggested that ammonium formate was the actual reactant, and it was therefore the object of this work to learn if there were conditions such that the reaction could be carried out with ammonium formate but not with formamide.



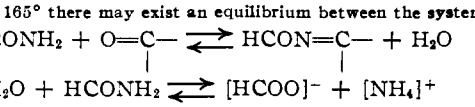
In Table I are summarized the important data from a number of reactions carried out under different conditions using acetophenone as the carbonyl component. In experiments 4 and 5 it will be observed that when the reaction was carried out in diethylene glycol solution at 120–130°, no reaction occurred with formamide even after as long as fifteen hours, while a yield of 10% was obtained with ammonium formate after four hours. Thus, although formamide is capable of reacting at temperatures greater than 165° (Experiments 1 and 2), at 120–130° ammonium formate appears to the reagent which is both necessary and sufficient for reaction.[2]

One mechanism for the reaction of a carbonyl compound with ammonium formate is the one originally proposed by Wallach[3] which is outlined in equations 3–5.



(1) (a) Goodson, Wiegand and Splitter, THIS JOURNAL, **68**, 2174 (1946); (b) Crossley and Moore, *J. Org. Chem.*, **9**, 529 (1944); (c) Novelli, THIS JOURNAL, **61**, 520 (1939); (d) Johns and Burch, *ibid.*, **60**, 919 (1938); (e) Ingersoll, Brown, Kim, Beauchamp and Jennings, *ibid.*, **58**, 1803 (1936).

(2) At 165° there may exist an equilibrium between the systems.



In support of this reaction series for the intermediate formation of ammonium formate, it will be observed that the yield was lowered from 30% to 17% by the introduction of anhydrous calcium sulfate into the reaction mixture (Experiments 1 and 2).

(3) Wallach, *Ann.*, **343**, 54 (1905).