of the solvent upon the rate constants is in fair agreement with the Scatchard–Christiansen theory.

5. The experimental results for this reaction are compared in methanol–water and in glycol–water mixtures.

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On the Mechanism of the Catechol–Tyrosinase Reaction. II. The Hydrogen Peroxide Question

BY CHARLES R. DAWSON AND BERNARD J. LUDWIG

The dihydric phenol catechol has been used widely as an experimental substrate in the study of phenolic–oxidase action. These studies, and the widespread occurrence of catechol derivatives in nature, have motivated the suggestion that the utilization of molecular oxygen during the respiration of certain types of plants probably is largely dependent on this type of enzymatic action.[1] Robinson and McCance[2] in 1925 were among the first to show that the enzymatic oxidation of catechol results in the absorption of two atoms of oxygen per molecule of the dihydric phenol. Although it has been established definitely that *o*-benzoquinone is one of the initial products formed during the action of a phenolic oxidase such as tyrosinase on catechol,[3–5] the nature of the enzymatic action and the chemical mechanisms involved have not yet been explained satisfactorily.

The conversion of catechol to *o*-benzoquinone is an oxidation requiring theoretically only one atom of oxygen, and thus much of the controversy during the past decade has been in reference to the fate of the second oxygen atom. Since aerobic oxidations are attended frequently by the initial formation of hydrogen peroxide, it is not surprising that one of the first mechanisms, as suggested by Onslow and Robinson,[6] accounted for the second atom of oxygen by the formation of hydrogen peroxide, *i. e.*

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

These investigators, and others,[7] have claimed to have detected traces of hydrogen peroxide in the reaction mixture resulting from the enzymatic oxidation of catechol, and they attributed the difficulty in detecting this substance to the presence of peroxidase and catalase in their tyrosinase or oxidase preparations. Others have attributed the difficulty of detecting hydrogen peroxide in many catalytic oxidations to the presence of small amounts of certain metals.[8,9]

The reliability of the methods used by Onslow and Robinson, and others,[7] for the detection of hydrogen peroxide has been questioned, and several workers have expressed doubt concerning the formation of hydrogen peroxide during the enzymatic oxidation of catechol. Pugh and Raper[4] studied the oxidation of catechol using tyrosinase preparations that possessed appreciable catalase activity, and observed that the total oxygen uptake did not deviate from two atoms per molecule of substrate. They pointed out that the presence of catalase would tend to decompose any hydrogen peroxide formed during the enzymatic oxidation and would thereby return the second atom of oxygen to the reaction mixture. These workers suggested that the second oxygen atom is consumed during further oxidation of *o*-benzoquinone. Furthermore, Nobutani[10] found that catalase has no effect on the oxygen consumption during the enzymatic oxidation of *p*-cresol, and therefore concluded that hydrogen peroxide is not formed during this reaction.

A study of the reports of Pugh and Raper and of Nobutani, however, reveals that their conclusions have been based on evidence that is not entirely convincing. These investigators did not indicate the strengths of the catalase preparations used nor did they demonstrate that the catalase remains active throughout the oxidation process.

(1) A. von. Szent-Györgyi, *Science*, **72**, 125 (1930).
(2) M. E. Robinson and R. A. McCance, *Biochem. J.*, **19**, 251 (1925).
(3) A. von Szent-Györgyi, *Biochem. Z.*, **162**, 399 (1925).
(4) C. B. M. Pugh and H. S. Raper, *Biochem. J.*, **21**, 1370 (1927).
(5) H. Wagreich and J. M. Nelson, *J. Biol. Chem.*, **115**, 459 (1936).
(6) M. W. Onslow and M. E. Robinson, *Biochem. J.*, **20**, 1138 (1926).
(7) B. S. Platt and A. Wormall, *ibid.*, **21**, 26 (1927).
(8) H. Wieland, "On the Mechanism of Oxidation," Yale University Press, New Haven, Conn., 1932.
(9) H. Wieland and W. Franke, *Ann.*, **457**, 1 (1927).
(10) F. Nobutani, *J. Biochem.* (Japan), **23**, 472 (1936).

Furthermore, it appears from some recent studies of Keilin and Hartree[11] that under certain conditions catalase may promote a secondary oxidation involving the hydrogen peroxide produced during an enzymatic oxidation. These authors have shown that when catalase functions in this capacity, the decomposition of the hydrogen peroxide does not result in the liberation of oxygen, *i. e.*, it does not return molecular oxygen to the reaction system. If, during the enzymatic oxidation of catechol, the introduction of catalase should promote such a secondary oxidation between hydrogen peroxide and some suitable intermediate, it is possible that the total oxygen uptake might not deviate from two atoms per molecule of substrate. Under such conditions, therefore, the formation of hydrogen peroxide would not be detected by oxygen uptake measurements.

In 1930 Pugh[12] reported that the activating action of traces of catechol and other dihydric phenols on the tyrosinase-monohydric phenol reaction could be attributed to the formation of either hydrogen peroxide or both hydrogen peroxide and *o*-quinone. More recently, Evans and Raper[13] have reported that the addition of purified peroxidase to the mealworm tyrosinase-tyrosine reaction causes a considerable decrease in the accumulation of dopa (3,4-dihydroxyphenylalanine). Since peroxidase itself has no action on mono- or dihydric phenols, they presented this as evidence that either hydrogen peroxide or an organic peroxide, which is capable of being activated by peroxidase, is produced in the tyrosinase–tyrosine reaction.

It has been found recently in these Laboratories[14] that the quinone produced during the enzymatic aerobic oxidation of catechol can be followed by iodimetric titration. When produced under certain conditions, *i. e.*, with an optimum amount of enzyme in very dilute solutions of catechol buffered in the *p*H range 4.2 to 6.6, the stability of the quinone is such that the stoichiometric relationship between it and catechol can be demonstrated. Since both quinone and hydrogen peroxide liberate iodine from hydriodic acid, it was apparent at an early stage in these studies that the indefinite status of the hydrogen peroxide controversy tended to cloud all interpretations. For this reason it became essential that more conclusive experimental evidence be obtained concerning the questionable role of hydrogen peroxide in this enzymatic oxidation.

In order to avoid the questionable reliability of the oxygen absorption methods which have been applied previously to this problem, the reaction course was followed by means of the iodimetric titration method previously described.[14] The study reported in this communication deals with the effect of hydrogen peroxide and the effect of catalase on the production and stability of the oxidation products formed during the catechol–tyrosinase reaction.

The Tyrosinase Preparations.—The tyrosinase preparations used in the present study were obtained from common mushrooms (common or field mushroom "*Psalliota campestris*"). The detailed procedure used for obtaining tyrosinase preparations from mushrooms will be published later. In brief, the mushrooms were frozen, ground, treated with cold acetone, and extracted with water. After an acetone precipitation and one or two ammonium sulfate precipitations, the enzyme was adsorbed on kaolin, eluted, and then adsorbed on alumina. The last step was followed by elution and dialysis, and the activity of the resulting preparation was measured according to the method proposed by Graubard and Nelson.[15]

The preparations were found to be free of peroxidase activity and to have negligible catalase activity. Typical data in support of this statement are compiled in Table I. These data were obtained using the procedure of Balls and Hale[16,17] for the determination of peroxidase activity.

The negligible magnitude of the catalase activity in the tyrosinase preparations can be judged by an inspection of the values of $(a - x)/t$ in column 1 of this table. The very small values of $(a - x)/t$ indicate a very low rate of decomposition of hydrogen peroxide by 5 cc. of the tyrosinase solution in the absence of pyrogallol. The absence of peroxidase activity in the tyrosinase solutions is shown by a comparison of the values of $(a - x)/t$ in columns 2 and 3 with those in column 1. The introduction of pyrogallol at

(11) D. Keilin and E. F. Hartree, *Proc. Roy. Soc.* (London), **B119**, 141 (1935).

(12) C. E. M. Pugh, *Biochem. J.*, **24**, 1442 (1930).

(13) W. C. Evans and H. S. Raper, *ibid.*, **31**, 2155 (1937).

(14) C. R. Dawson and J. M. Nelson, THIS JOURNAL, **60**, 250 (1938).

(15) M. Graubard and J. M. Nelson, *J. Biol. Chem.*, **112**, 135 (1935).

(16) A. K. Balls and W. S. Hale, *J. Assoc. Off. Agr. Chem.*, **16**, 445 (1933).

(17) A. K. Balls and W. S. Hale, *J. Biol. Chem.*, **107**, 767 (1934).

TABLE I

250 cc. of 0.02 *M* phosphate buffer contained 4.0 cc. of 0.1 *N* H_2O_2 with enzyme and pyrogallol as shown. The solution was covered with oil to prevent access of air and was stirred with nitrogen. Titrations were made on 25-cc. portions with thiosulfate solution (approximately 0.01 *N*). The value of $(a - x)/t$ gives a measure of the rate of decomposition of the hydrogen peroxide.

	*p*H 6.6 5 cc. tyrosinase soln. 56.5 units No pyrogallol		*p*H 6.6 5 cc. tyrosinase soln. 56.5 units 0.6 g. pyrogallol		*p*H 7.8 5 cc. tyrosinase soln. 56.5 units 0.6 g. pyrogallol		*p*H 7.8 5 cc. peroxidase diluted (1:20) 0.6 g. pyrogallol		*p*H 7.8 5 cc. peroxidase diluted (1:20) 0.6 pyrogallol 5 cc. tyrosinase 56.5 units	
Time, min.	Thio., cc.	$\frac{a-x}{t}$	Thio., cc.	$\frac{a-x}{t}$	Thio., cc.	$\frac{a-x}{t}$	Thio., cc.	$\frac{a-x}{t}$	Thio., cc.	$\frac{a-x}{t}$
0	4.07		4.10		4.42		4.06		3.91	
2	4.03	0.02	4.05	0.02	4.40	0.01	3.70	0.18	3.56	0.18
5	4.00	.01	4.00	.02	4.39	.01	3.19	.17	3.06	.17
8	3.93	.02	3.92	.02	4.37	.01	2.71	.17	2.60	.16
10	3.86	.02	3.85	.02	4.33	.01	2.43	.16	2.32	.16

*p*H 6.6 did not increase the rate of decomposition of hydrogen peroxide, and at *p*H 7.8 caused a slightly lower rate of decomposition. Furthermore, as shown in columns 4 and 5, a mixture of 5 cc. of the tyrosinase solution with 5 cc. of a diluted horse radish peroxidase preparation had the same peroxidase activity as the peroxidase preparation alone.

The Effect of Hydrogen Peroxide on the Stability of the Oxidation Product Formed During the Enzymatic Oxidation of Catechol.—In dilute solutions buffered within the *p*H range 4.2 to 6.6 and in the presence of sufficient tyrosinase, catechol is rapidly and completely converted by air to an oxidized state that is equivalent to two atoms of iodine per molecule of catechol.[14] This iodine equivalency naturally suggests the oxidation product to be a benzoquinone, but does not preclude the possibility that the titration values may correspond to a mixture of a benzoquinone and hydrogen peroxide. Within this *p*H range the decrease in stability of the oxidation product with increase in *p*H is small, as can be seen in curves 1, 2 and 3 of Fig. 1, but at higher *p*H values the product is too unstable to be determined effectively. Since the tyrosinase preparations used in these experiments possess negligible catalase and peroxidase activity, hydrogen peroxide, if formed during the oxidation of catechol under these conditions, should be detectable, provided that the hydrogen peroxide does not undergo a secondary reaction with the benzoquinone.

To determine the conditions favoring such a secondary reaction between the benzoquinone and hydrogen peroxide, the enzymatic oxidation of catechol in the presence of given amounts of hydrogen peroxide at different hydrogen ion concentrations were studied. The results obtained are shown graphically in curves 5, 6 and 7 of Fig. 1. The experimental conditions and other points of information are given in the legend of this figure. As explained in this legend, 1.91 cc. of the thiosulfate solution is the titration value that represents complete conversion of 10 mg. of catechol to the quinone state, *i. e.*, to an oxidized state that is equivalent to two atoms of iodine per molecule of catechol. Thus, in the presence of an amount of hydrogen peroxide equivalent to 3.30 cc. of the thiosulfate solution per titration sample (see curve 4, Fig. 1), the complete enzymatic conversion of 10 mg. of catechol to this quinone state should be represented by a titration value of 5.21 cc. of thiosulfate solution, provided the hydrogen peroxide and quinone are perfectly compatible. Curves 6 and 7 of this figure show the quinone and hydrogen peroxide to be incompatible in solutions buffered to *p*H values of 5.5 and 6.7, respectively. However, at *p*H 4.1 hydrogen peroxide appears to have very little effect on the stability of the quinone produced in this enzymatic oxidation, as can be judged from a comparison of curve 5 with curve 1 in this figure. Since hydrogen peroxide and the quinone appear to be compatible at *p*H 4.1, it is evident that experiments designed for the detection of hydrogen peroxide as a reaction product in this enzymatic oxidation should be conducted in solutions buffered to approximately this *p*H value.

The Effect of Catalase on the Reaction Course as Determined by the Iodimetric Titration Method.—By the use of a sufficient amount of the enzyme catalase, the decomposition of hydrogen peroxide can be effected at a rate so rapid as to be practically immeasurable. For this reason, the presence of a relatively large amount of catalase in the reaction system, during the enzymatic

oxidation of catechol at *p*H 4.1, should alter materially the reaction course, as determined by the iodimetric titration method, if hydrogen peroxide is responsible for any part of the titration values.

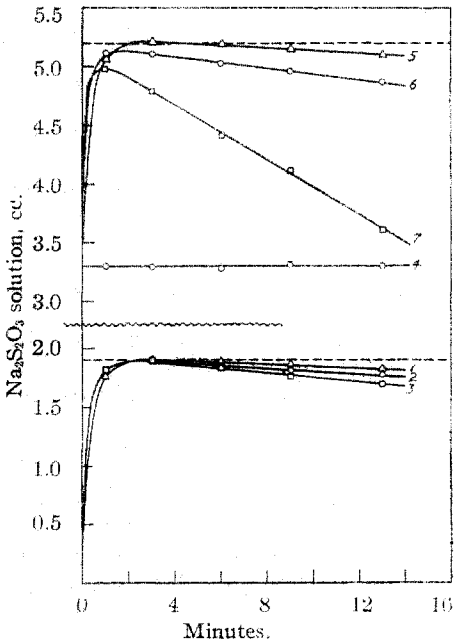

Fig. 1.—Curves showing the effect of *p*H and of hydrogen peroxide on the production and stability of the oxidation product formed in the catechol–tyrosinase reaction. 3.5 units of tyrosinase per mg. of catechol was used in solutions buffered to the following *p*H values: Curve 1, *p*H 4.1; 2, *p*H 5.3; 3, *p*H 6.7; 5, *p*H 4.1; 6, *p*H 5.5; 7, *p*H 6.7. The enzymatic oxidations represented by curves 1, 2 and 3 were run at 25° in a total reaction volume of 250 cc., of which 25 cc. was 0.2 *M* phosphate buffer; 5 cc. was 0.2% freshly prepared catechol solution (10 mg.), and 5 cc. was enzyme solution. The enzyme was added at zero time. With air bubbling through the reaction solution, 25-cc. samples were removed at the time intervals noted and added to 25 cc. of 2 *N* sulfuric acid. After adding 10 cc. of 10% potassium iodide they were allowed to remain in the dark for fifteen minutes before titrating the liberated iodine with standard thiosulfate solution. Thus the plotted volumes of thiosulfate solution required to take care of the iodine liberated in each sample are a measure of the amounts of oxidation product causing this iodine liberation. 1 Mg. of catechol per 25 cc. (0.1 reaction volume) when completely oxidized is equivalent to 9.10×10^{-6} mole of iodine which in turn is equivalent to 1.91 cc. of 0.00950 *N* sodium thiosulfate solution. The runs represented graphically by curves 5, 6 and 7 differ from those of curves 1, 2 and 3 in that the reaction system corresponding to the upper curves contained in addition to 10 mg. of catechol and 35 units of enzyme 2.0 cc. of approximately 0.15 *N* hydrogen peroxide. The titration values plotted in curve 4 (3.30 ± 0.01 cc.) are those obtained when the system contained only this amount of hydrogen peroxide. The upper dotted line (at 5.21 on the ordinate axis) is the sum of the hydrogen peroxide equivalent (3.30) and the catechol equivalent (1.91) in cc. of 0.00950 *N* sodium thiosulfate solution.

Curves 1 and 2 in Fig. 2 show the reaction course, as determined by this method, when 10 mg. of catechol in 250 cc. of solution buffered to *p*H 4.1 was oxidized rapidly with 35 units of tyrosinase and more slowly oxidized with 5 units of the enzyme. Curves 4 and 5 show the results obtained when the experiments were repeated

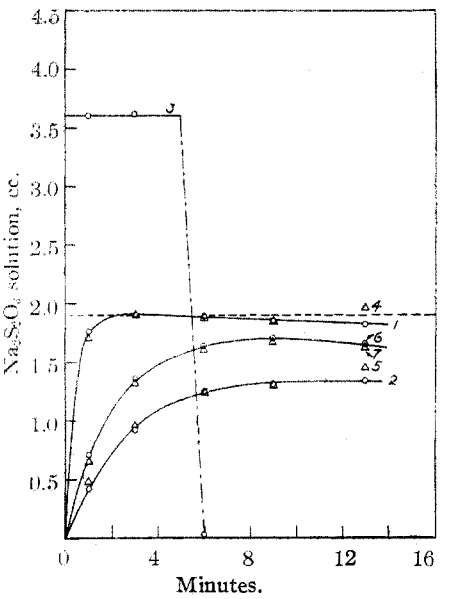

Fig. 2.—Curves showing the absence of hydrogen peroxide in the products resulting from the enzymatic oxidation of catechol. The data represented in curves 1, 2, 4 and 5 were obtained using 10 mg. of catechol per 250 cc. reaction volume (buffered to *p*H 4.1) according to the procedure described in the legend of Fig. 1. The data represented in curves 6 and 7 were obtained with similar solutions buffered to *p*H 5.6. Tyrosinase used per mg. of catechol: curves 1 and 4, 3.5 units; curves 6 and 7, 1.0 unit; curves 2 and 5, 0.5 unit. The systems represented by curves 4 and 5 differ from those of curves 1 and 2 only in that the initial reaction volume (250 cc.) of the former systems contained, in addition to catechol and tyrosinase, 2.0 cc. of a diluted catalase preparation. The data represented in curve 3, which has been included to give an indication of the catalase activity possessed by this amount of the catalase preparation, were obtained by use of the same titration procedure. The system contained initially only 2.0 cc. of approximately 0.17 *N* hydrogen peroxide in 250 cc. This amount of hydrogen peroxide is nearly twice that theoretically producible in the enzymatic oxidation of catechol under these conditions. As can be seen in this curve, one minute after the addition of 2.0 cc. of the diluted catalase preparation, only a negligible quantity of hydrogen peroxide remained. To demonstrate that the catalase remained active throughout the oxidation process, a similar amount of hydrogen peroxide (2.0 cc. of 0.17 *N* hydrogen peroxide) was added after the expiration of twelve minutes to the systems represented by curves 4 and 5. Had the catalase been inactivated completely the last titration value in each of these curves would have been greater by about 5.8 cc. of the thiosulfate solution. The system represented by curve 7 differs from that of curve 6 only in that the former contained initially 5 cc. of a diluted (1:20) peroxidase preparation in addition to the catechol and tyrosinase. The activity of this amount of peroxidase can be judged from an inspection of the data in column 4 of Table I. The titrations in the experiments represented by curves 6 and 7 were made with 0.00964 *N* sodium thiosulfate.

in the presence of a relatively large amount of catalase. This negative effect of catalase on the reaction course was observed also at other *p*H values within the *p*H range 4.1 to 6.7. Experiments of this type were also conducted at several *p*H values using peroxidase rather than catalase for the purpose of detecting hydrogen peroxide. In no case did the presence of peroxidase alter the reaction course. A typical example is presented in the form of curves 6 and 7 of Fig. 2 (see

legend). As explained in the legend and shown in curve 3 of this figure, the amount of catalase used in these studies was sufficient to decompose in about one minute nearly twice the amount of hydrogen peroxide that theoretically could be produced during this enzymatic oxidation. Since the catalase remained active throughout the enzymatic oxidation (see legend of Fig. 2) and caused no change in the course of the reaction as followed by this method, it can be concluded definitely that the titration values are in no part due to hydrogen peroxide.

Discussion

It is quite apparent that the uptake of the extra atom of oxygen over and above that which is required for the formation of *o*-benzoquinone from catechol cannot be accounted for in this enzymatic oxidation simply by the formation of hydrogen peroxide as suggested by Onslow and Robinson,[6] *i. e.*

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

This statement, based largely on the evidence that has been presented above, has recently received further substantiation during the course of other investigations in these Laboratories. By simultaneously following the enzymatic oxidation of catechol with both oxygen uptake measurements and iodimetric titration measurements, Wagreich and Nelson[18] have found that the complete conversion of catechol to the quinone state, as indicated by iodimetric titration, requires the absorption of only one atom of oxygen. It appears quite probable that the second oxygen atom is used in oxidation of a product resulting from the action of water on *o*-benzoquinone.

The question of whether or not hydrogen peroxide has an intermediary function during the formation of the initial quinone may be considered to be still open to debate. For such to be the case, the hydrogen peroxide must perform this function at a rate greater than the rate of its own decomposition by relatively large amounts of catalase, and its concentration therefore at any given time must be extremely low. If the tyrosinase preparations had possessed appreciable peroxidase activity, such an intermediary function of hydrogen peroxide would be more readily conceivable. Considering the facts that have been presented above, there appears to be little reason at present for supporting such a view of the enzymatic oxidation of catechol.

(18) H. Wagreich and J. M. Nelson, THIS JOURNAL, **60**, 1545 (1938).

Summary

1. The enzymatic oxidation of catechol has been studied using tyrosinase preparations having no peroxidase activity and negligible catalase activity.

2. In dilute solutions buffered within the *p*H range 4.1 to 6.7 the oxidation product formed in the initial stages of the catechol–tyrosinase reaction is equivalent to two atoms of iodine per molecule of catechol. This iodine equivalency corresponds to a benzoquinone.

3. This quinone and hydrogen peroxide are incompatible in solutions buffered to *p*H values above 4.1.

4. In the *p*H range 4.1 to 6.7, the course of the catechol–tyrosinase reaction, as followed by iodimetric titration, is unaltered by large amounts of catalase or peroxidase.

5. It is concluded that hydrogen peroxide is not responsible for any part of the titration values, and that the consumption of two atoms of oxygen per molecule of catechol during the enzymatic oxidation cannot be directly attributed to the formation of hydrogen peroxide.

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