possible loss of phenol vapor, which is appreciable under the reduced pressure. To minimize, in the subsequent bromination of the phenol, the loss of bromine and to prevent[15] the bromination of any catechol still present, the last-mentioned mixture was cooled to −3° by the addition of 500 g. of chopped ice (prepared from distilled water). To the cold mixture 12.2 cc. of 0.1 *M* potassium bromate solution and 10 cc. of 15% potassium bromide solution were added and the mixture allowed to stand for twenty minutes. After the bromination had taken place, 10 cc. of a 25% solution of potassium iodide was added and the mixture allowed to stand in a dark place for fifteen minutes, after which the liberated iodine was determined by means of 0.1 *M* sodium thiosulfate solution.

In determinations involving small amounts of phenol (30 to 35 mg.) in the presence of large amounts of the final oxidation product the procedure was modified by using 5 cc. instead of 3 of the gelatin solution and twice as much lead acetate solution.

(15) Francis and Hill, THIS JOURNAL, **46**, 2498 (1924).

The results of analyses made on prepared mixtures of phenol and catechol oxidation products had an average deviation of ±0.7%. The quantities of phenol found to be present at any time in the experiments corresponding to Curves I and II in the figure were therefore reliable to ±0.7%.

Summary

1. A method has been devised for determining the activity of tyrosinase toward phenol by determining the rate of disappearance of phenol from the reaction mixture.

2. A comparison of the direct phenol method with the oxygen uptake method, which is usually used, shows that the latter method is reliable for estimating the monohydric phenol activity of tyrosinase toward phenol.

NEW YORK, N. Y. RECEIVED JANUARY 22, 1944

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

The Aerobic Oxidation of Phenol by Means of Tyrosinase

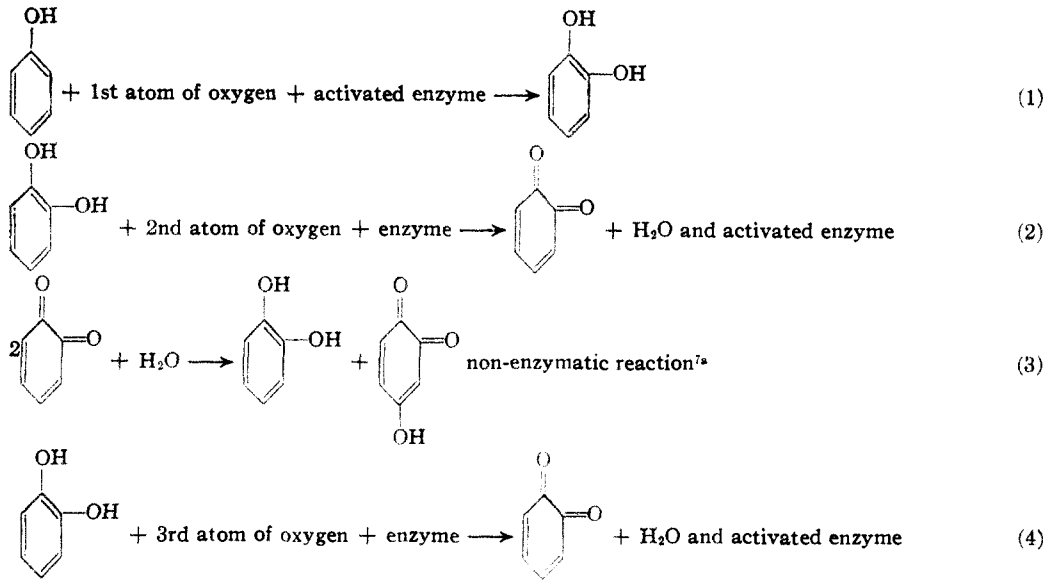
BY RALPH C. BEHM AND J. M. NELSON

When monohydric phenols, such as *p*-cresol,[1] phenol or *l*-tyrosine[2] are aerobically oxidized by means of tyrosinase, the reactions usually start with an initial lag. This induction period is overcome by the addition of a small amount of an *o*-dihydric phenol, such as catechol, to the reaction mixture.[1,2,3] Several explanations have been suggested as to how the *o*-dihydric phenol serves to decrease the induction period.[2,3,4] Since the enzyme also catalyzes the oxidation of *o*-dihydric phenols to their corresponding *o*-quinones, most of these explanations have been based on the view that the monohydric phenols are oxidized by the quinones. Bordner and Nelson,[1] however, have shown that the oxidation of the monohydric phenols to the *o*-dihydric state cannot be attributed to the quinones.

A theory for the oxidation of monohydric phenols by means of tyrosinase, which has several experimental facts in its favor, has been gradually taking shape in these laboratories. It is based on the idea that for tyrosinase to catalyze the oxidation of monohydric phenols, it must first be activated toward monohydric phenols, by simultaneously oxidizing an *o*-dihydric phenol.[1,5] This theory can be expressed briefly by reactions represented as shown beyond.

Some experimentally established facts, supporting the theory, are listed below and in the case of some of these a brief explanation has been added: (1) The fact that when phenol is enzymatically oxidized three atoms of oxygen are consumed per molecule of phenol oxidized is taken care of. (2) The addition of catechol decreasing the induction period and enabling the enzyme to catalyze the oxidation of phenol is accounted for by reactions (1) and (2). (3) Adding oxidizing agents, such as potassium ferricyanide, or the phenol oxidase, laccase, at the beginning of the reaction when phenol is oxidized by the enzyme lengthens the induction period.[1] These oxidizing agents would tend to deprive the enzyme of oxidizing catechol (reaction 2) and hence decrease the activation of the enzyme toward phenol. Less activation at the beginning of the reaction would slow down reaction (1) and lengthen the induction period. (4) Benzenesulfinic acid added initially to the reaction mixture when *p*-cresol or phenol is oxidized by the enzyme greatly lengthens the induction period.[1,6] Benzenesulfinic acid combines with *o*-quinone[1,6] and consequently would eliminate reaction (3). Elimination of reaction (3) would mean less catechol being formed and oxidized and hence less activation. Less activation, as pointed out in 3, would lengthen the induction period. (5) The length of the induction period has been found to decrease with increase in *p*H.[1] The *o*-quinone disappears faster as its aqueous solution becomes more alkaline.[7] The latter means the rate of reaction (3) increases with increase in *p*H and hence catechol is returned to the solution and oxidized faster. As a result the enzyme is activated faster, the rate of reaction (1) increased and the induction period shortened. (6) In the present study it has been found that the addition of a reducing agent such as ascorbic acid shortens the induction period. Since the

(1) Bordner and Nelson, THIS JOURNAL, **51**, 1507 (1939).
(2) Califano and Kertesz, *Nature*, **142**, 1036 (1938).
(3) Onslow and Robinson, *Biochem. J.*, **22**, 1327 (1928).
(4) Richter, *ibid.*, **28**, 901 (1934).
(5) Gregg and Nelson, THIS JOURNAL, **62**, 2506 (1940).
(6) Pugh and Raper, *Biochem. J.*, **21**, 1370 (1927).
(7) Dawson and Nelson, THIS JOURNAL, **60**, 245 (1938).



ascorbic acid reduces the *o*-quinone formed in reaction (2), more catechol would be formed and oxidized. As a result the enzyme would be activated faster and increase the rate of reaction (1).

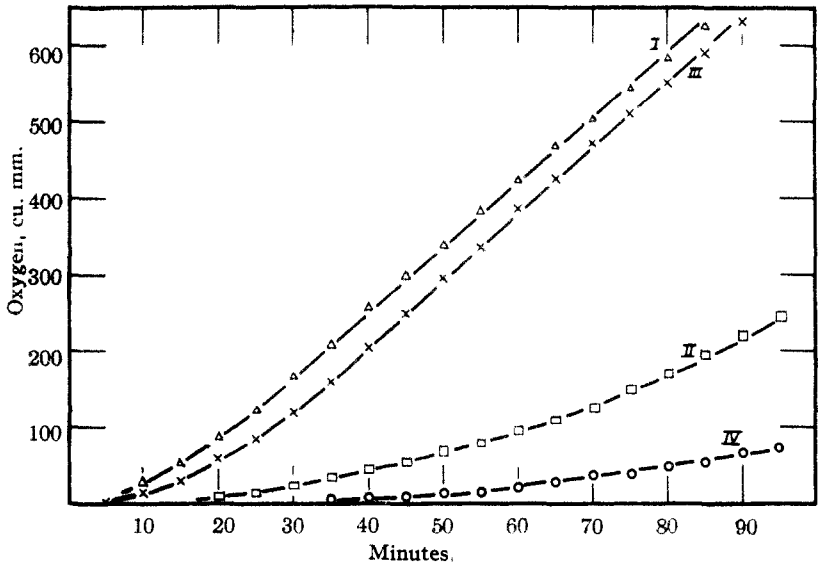


Fig. 1.—Showing that the oxidation of phenol by means of tyrosinase is more dependent on the oxidation of *o*-dihydric phenol than is the oxidation of *p*-cresol: Warburg respirometer; temperature 25°; *p*H 7.0. Reaction mixture for Curve I: 4 cc. of *M*/15 phosphate buffer, 1 cc. of gelatin solution (5 mg. of gelatin), 2 cc. of *p*-cresol (8 mg. *p*-cresol), and 1 cc. of mushroom tyrosinase containing 0.85 *p*-cresolase unit.[12] Reaction mixture Curve II: same as for Curve I, except 4 cc. of *M*/15 sodium borate buffer used in place of the phosphate buffer (final *p*H 7). Reaction mixtures for Curves III and IV, similar to those for Curves I and II, respectively, except 2 cc. of a phenol solution (8 mg. of phenol) was used in place of the *p*-cresol.

In other words, the induction period would be shortened. (7) When 4,5-dimethylphenol (xylenol) is oxidized by means of tyrosinase the induction period is long.[8] Due to positions 4 and 5 being occupied, the 4,5-dimethylquinone cannot undergo reaction (3) and less of the catechol compound is returned to the reaction mixture than when phenol constituted the substrate. The oxidation of less *o*-dihydric phenol amounts to less activation, slower rate for reaction (1) and consequently a longer induction period. (8) The fact that no preparation of tyrosinase, free of *o*-dihydric phenol activity, has ever been prepared also can be taken as evidence supporting the above theory.

That the activation of tyrosinase toward monohydric phenols is transitory and requires the simultaneous oxidation of an *o*-dihydric phenol, *i. e.*, reaction (1) can only occur if at the same time reaction (2) is taking place, is indicated by the following experiment. A hundred times the amount of catechol necessary to prevent the induction period was first oxidized completely by the enzyme (catechol is oxidized very rapidly by tyrosinase[9]). Immediately afterward phenol was added. The enzymatic oxidation of the phenol, however, started

(7a) Wagreich and Nelson, THIS JOURNAL, **60**, 1545 (1938).

(8) Cushing, Dissertation, Columbia University, 1942.

(9) Nelson, *Cold Spring Harbor Symposia on Quantitative Biology*, **7**, 148 (1939).

with a lag period showing that the activation caused by the previous oxidation of the catechol had disappeared.

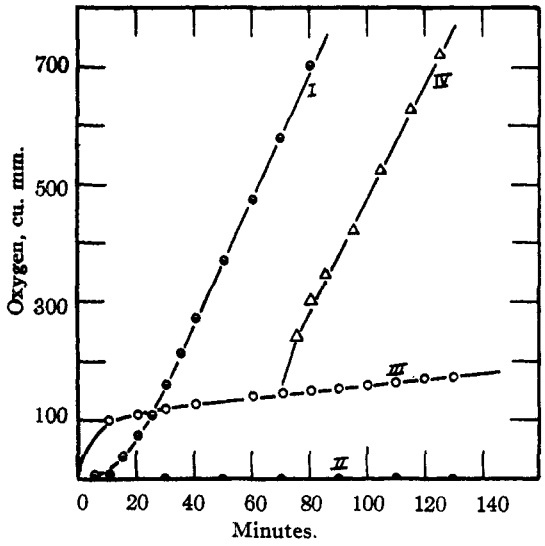


Fig. 2.—Showing that the oxidation of one molecule of catechol per molecule of phenol oxidized does not activate tyrosinase sufficiently to enable the reaction to overcome the induction period, nor to increase the existing rate of oxidation when an excess of phenol–enzyme complex is present in the reaction mixture. Warburg respirometer was used[10]; temp. 25°. Curve I represents the rate of oxygen uptake (rate of phenol disappearance from the reaction mixture[11]) when phenol was oxidized by means of tyrosinase. Reaction mixture for Curve I: 1 cc. of phenol solution (5 mg. of phenol), 2 cc. of $M/15$ phosphate buffer (*p*H 6.7), 1 cc. of gelatin solution (0.2 mg.),[11a] 1 cc. of a high catecholase preparation of mushroom tyrosinase containing 1.1 phenolase[12] and 22.8 catecholase[13] units[13a] and sufficient water to make the final reaction volume equal to 8 cc. Curve II shows that benzene sulfinic acid, initially present, prevents the enzymatic oxidation of the phenol. Reaction mixture for Curve II: same as for Curve I, except it also contained 3 mg. of sodium benzene sulfinate. Curve III shows that the addition of a small amount of catechol overcomes the inhibiting action of the benzenesulfinic acid. Reaction mixture for Curve III: same as for Curve II, except 0.2 mg. of catechol was added at zero time.[14a] The addition of 20 mg. of phenol to the reaction mixture corresponding to Curve III, after the reaction had been in progress for seventy minutes, failed to increase the rate of oxygen uptake. Although the concentration of the phenol–enzyme complex must have been increased considerably when the 20 mg. of phenol was added, the presence of the sulfinic acid, preventing reaction (3) from taking place, left only the one molecule of catechol, formed in reaction (1), to be oxidized per molecule of phenol oxidized. The oxidation of this one molecule of catechol was only able to activate the enzyme to such an extent that the existing rate of oxygen uptake could continue. As soon, however, as 0.2 mg. of catechol was added with the 20 mg. of phenol, after the reaction had been in progress for seventy minutes, enough catechol was oxidized, per molecule of phenol oxidized, to raise the rate of oxygen uptake to the maximum rate, Curve IV, determined by the enzyme operating at a phenol concentration equivalent to the optimum phenol concentration.

As already mentioned, the influence of benzenesulfinic acid, initially present, on the oxidation of phenol prevents the reaction from starting, but only lengthens the induction period when *p*-cresol is the substrate. This difference shown by the two monohydric phenols suggests that the enzyme requires more activation, *i. e.*, a greater rate of *o*-dihydric phenol oxidation (reaction (2)) in the case of the enzymatic oxidation of phenol than when *p*-cresol is being oxidized. That such is actually the case is indicated by the results shown in Fig. 1. The two monohydric phenols were oxidized by means of the enzyme in the presence and absence of boric acid. It will be noticed that the oxidation of the phenol was retarded more by the boric acid than that of the *p*-cresol. It is well known that boric acid tends to combine with *o*-dihydric phenols and therefore would tend to reduce the amount of *o*-dihydric phenol oxidized in reaction (2). If the phenol oxidation requires more activation of the enzyme than does the *p*-cresol oxidation, then it follows that the presence of the boric acid would affect the phenol oxidation the most.

The data in Fig. 2 show that the oxidation of one molecule of catechol (reaction (2)) per molecule of phenol oxidized only permits the enzymatic oxidation of phenol to proceed at the existing rate. When benzenesulfinic acid is present at the beginning of the reaction and the rate of the phenol oxidation is zero, the rate remains zero (Curve II). On the other hand, if the reaction is progressing in the presence of the sulfinic acid and the rate is not zero (Curve III) then the rate will continue at this rate, even though the concentration of the phenol–enzyme complex is increased by the addition of a large amount of phenol to the reaction mixture. The latter rate of oxygen uptake can only be increased by also adding a small amount of catechol (Curve IV). This constant rate of phenol oxidation, in the presence of benzene sulfinic acid, irrespective of the concentration of the phenol–enzyme complex, indicates that the one molecule of catechol formed in reaction (1) (reaction (2) being prevented from taking place by the sulfinic acid) does not give rise to sufficient activation to increase the existing rate of oxidation of the phenol. On the other hand (see Fig. 4, Bordner and Nelson[1]),

(10) Dixon, "Manometric Methods," Cambridge University Press, 1934.

(11) Behm and Nelson, THIS JOURNAL, **66**, 709 (1944).

(11a) Gelatin used to stabilize the enzyme preparation.

(12) Adams and Nelson, THIS JOURNAL, **60**, 2472 (1938).

(13) Miller, Mallette, Roth and Dawson, *ibid.*,, **66**, 514 (1944).

(13a) The enzyme preparation had an optimum phenol concentration of less than 5 mg. of phenol per 8 cc. reaction mixture.[14] The phenolase units were determined by the same method as used for cresolase units.

(14) Gregg and Nelson, THIS JOURNAL, **62**, 2506 (1940).

(14a) Besides removing the *o*-benzoquinone, as it is formed in reaction (2), the sulfinic acid also competes with the phenol for the enzyme, *i. e.*, it is a competitive inhibitor as shown in Fig. 3. Therefore, the rate of oxygen uptake shown by Curve III, after the induction period (zero rate) was overcome by the addition of the 0.2 mg. of catechol, is the maximum rate. The reason the maximum rate (Curve III) was much less than the maximum rate shown by Curve I was due to the sulfinic acid, as a competitive inhibitor, lowering the concentration of the phenol–enzyme complex.

when *p*-cresol is oxidized in the presence of sulfinic acid the oxidation of one molecule of homocatechol per molecule of *p*-cresol oxidized (Reaction (2)) causes enough activation of the enzyme to permit the rate of oxidation to gradually increase and overcome the induction period, although more slowly than when no sulfinic acid is present.

The question may arise: How can tyrosinase initiate the oxidation of a monohydric phenol, if it first has to be activated by catalyzing the oxidation of an *o*-dihydric phenol? The induction period shows that some product is formed autocatalytically which enables the oxidation of the monohydric phenol to gradually attain the maximum rate. The fact that the addition of a trace of catechol immediately overcomes the induction period suggests that phenol is first oxidized to catechol and that the rate of formation of the latter autocatalytically increases as the reaction progresses. Probably the phenol used contained a few molecules of catechol, or the latter are formed by slow autoxidation when the reaction mixture is agitated with air at the beginning of the reaction. As pointed out above, the oxidation of one molecule of an *o*-dihydric phenol, per molecule of monohydric phenol oxidized, is sufficient to permit the *p*-cresol reaction to slowly overcome its induction period but only keeps the phenol oxidation continuing at the existing rate. It therefore follows that the oxidation of the two molecules of *o*-dihydric phenol, formed in reactions (1) and (3), increases the activation of the enzyme to a greater degree and permits the reaction to attain its maximum rate.

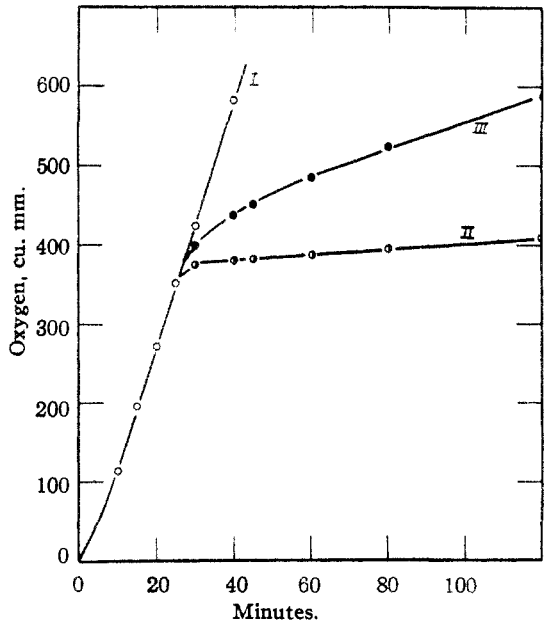


Fig. 3.—Showing that benzenesulfinic acid behaves as a competitive inhibitor toward phenol in the aerobic oxidation of phenol by means of tyrosinase. Curve I shows the rate of oxygen uptake for a reaction mixture consisting of 2 cc. of *M*/15 phosphate buffer *p*H 6.7, 1 cc. of phenol solution (20 mg.), 1 cc. of gelatin solution (0.2 mg.), 1 cc. of a high catecholase preparation containing 1.3 phenolase[12] units and 28.3 catecholase units,[13] and sufficient water to make the total volume in the reaction vessel 8 cc. The same curve was obtained if 1 cc. of phenol solution contained only 5 mg. of phenol. In each case the reaction had been in progress for ten minutes before the manometers of the Warburg respirometer were closed at "zero" time. Curve II shows the effect of addition of 35 mg. of sodium benzenesulfinate at time = 25 minutes to a reaction mixture similar to that for Curve I, using 5 mg. of phenol. Curve III shows the effect of addition of 35 mg. of sodium benzenesulfinate at time = 25 minutes to a reaction mixture similar to that for Curve I, using 20 mg. of phenol. The slope of the constant rate achieved after the addition of sulfinate in the experiment represented by Curve II (using 5 mg. of phenol) is 0.38 cu. mm. of oxygen uptake per minute; that of the experiment represented by Curve III (using 20 mg. of phenol) is 1.67 cu. mm. of oxygen uptake per minute. The change in slope, caused by the fourfold variation in phenol concentration in the presence of a constant amount of sodium benzenesulfinate, is 4.4. In view of the fact that experience in these laboratories has shown that benzenesulfinate has practically no effect on the rate of the enzymatic oxidation of catechol (the primary agent in this system) the retarding effect of the sulfinate on the phenol oxidation seems best explained on the basis that it displaces phenol from the enzyme; or in other words, benzenesulfinic acid behaves as a competitive inhibitor for phenol in the aerobic oxidation of phenol by means of tyrosinase.

Concerning the nature of the activation of tyrosinase toward monohydric phenols caused by the enzymatic oxidation of an *o*-dihydric phenol little is known. As shown by Bordner and Nelson, the oxidation of the monohydric phenols cannot be attributed to quinones or hydrogen peroxide formed in the oxidation of the *o*-dihydric phenols. That any other oxidation product of the *o*-dihydric phenols is responsible for the monohydric phenol also seems to be excluded as is indicated by the following experiment. Phenol was oxidized by means of the enzyme in the absence and presence of a relatively large amount of catechol and the rate of phenol disappearance from the reaction mixture followed in both instances by the method of Behm and Nelson.[11] The results indicated that the oxidation of the added catechol exerted practically no influence on the rate of disappearance of the phenol, except to eliminate the induction period.

Summary

Additional evidence is presented supporting the view that for tyrosinase to catalyze the oxidation of a monohydric phenol, the enzyme must catalyze simultaneously the oxidation of an *o*-dihydric phenol.

To overcome the induction period, when phenol is acted on by tyrosinase, it is necessary for the enzyme to catalyze simultaneously the oxidation of more than one molecule of catechol per molecule of phenol oxidized.

To increase the existing rate in the oxidation of phenol, when the latter is lower than the maximum, it is necessary to oxidize simultaneously more than one molecule of an *o*-dihydric phenol per molecule of phenol oxidized.

NEW YORK, N. Y. RECEIVED FEBRUARY 9, 1944