reaction in 99% methanol of potassium iodide with monosubstituted ethylene dibromides, $RCHBrCH_2Br$ where R was C_3H_7, C_6H_5, $C_6H_5CH_2$, CH_2OH, COOH and Br.

It is shown that, since each of the bromine atoms can react and at a different rate and with a different heat of activation, no theoretical significance can be given to the effect of various substituents, R, on the observed values of the heats of activation and entropies of activation of the reaction of $RCHBrCH_2Br$ with potassium iodide.

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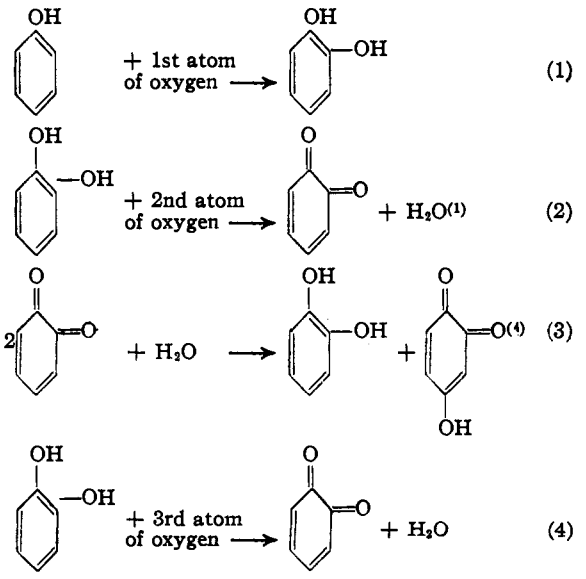
[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

The Activity of Tyrosinase toward Phenol

BY RALPH C. BEHM AND J. M. NELSON

As shown by several investigators[1,2,3] the phenol oxidase, tyrosinase, possesses two essentially different enzymatic activities. It not only catalyzes the aerobic oxidation of many *o*-dihydric phenols to their corresponding *o*-quinones, but also catalyzes the introduction of a second hydroxyl group into several monohydric phenols, *ortho* to the hydroxyl group already present. These oxidation reactions are not simple, but involve several consecutive and concurrent reactions, especially when monohydric phenols constitute the substrate. Taking phenol and catechol as representing the two types of phenols, the final oxidation product in both instances is *p*-hydroxyquinone.[4] In the enzymatic oxidation of phenol at *p*H 6, 3 atoms of oxygen[5] are consumed per molecule of phenol oxidized.



The method usually used for estimating the activity of tyrosinase toward a monohydric phenol is based on determining the rate of oxygen uptake by means of a Warburg or Barcroft respirometer.[6] It is apparent, however, from what has been stated above, that only the first atom of oxygen is concerned with the enzyme's action on the monohydric phenol. The remaining two atoms of oxygen are consumed in the enzyme's action on the *o*-dihydric phenol. The question therefore arises: Can the rate of oxygen uptake serve as a true measure of the enzyme's activity toward a monohydric phenol?

To answer the latter question, it was decided to determine, instead of the rate of oxygen uptake, the rate of disappearance of the monohydric phenol from the reaction mixture. Such a method would be independent of all the accompanying reactions encountered in the oxygen uptake method. For the purpose a reaction mixture, consisting of the enzyme, phenol, buffer and water, was placed in a reaction vessel and air bubbled through the mixture. Samples were removed at regular time intervals and their phenol contents determined. The results obtained are shown as Curve I. Curve II represents the rate of oxygen uptake for a reaction mixture, identical in composition with the one used in following the rate of phenol disappearance. It will be observed that the two curves coincide within experimental error, showing that, at least when phenol is the monohydric phenol being oxidized by means of the enzyme, the concurrent reactions, equations (2), (3) and (4) must proceed at rates at least as great as the rate of the initial oxidation of the phenol (equation 1) and that, therefore, the rate of oxygen uptake can serve as a true measure of the enzyme's activity toward phenol.

Tyrosine preparations, depending on the method of preparation, often vary widely with respect to their relative activities toward monohydric and *o*-dihydric phenols. Therefore, it has become customary, in these Laboratories, to designate a preparation low in activity toward *p*-cresol compared to its activity toward catechol as a high catecholase preparation and a preparation high in *p*-cresolase activity as a high cresolase preparation.[7] Since in the present study phenol was used instead of *p*-cresol, it should be men-

(1) Pugh and Roper, *Biochem. J.*, **21**, 1370 (1927).
(2) Dalton and Nelson, THIS JOURNAL, **61**, 2946 (1939).
(3) Bordner and Nelson, *ibid.*, **61**, 1507 (1939).
(4) Wagreich and Nelson, *ibid.*, **60**, 1545 (1938).
(5) Unpublished results obtained in these Laboratories.
(6) Adams and Nelson, THIS JOURNAL, **60**, 2472 (1938).
(7) Parkinson and Nelson, *ibid.*, **62**, 1693 (1940).

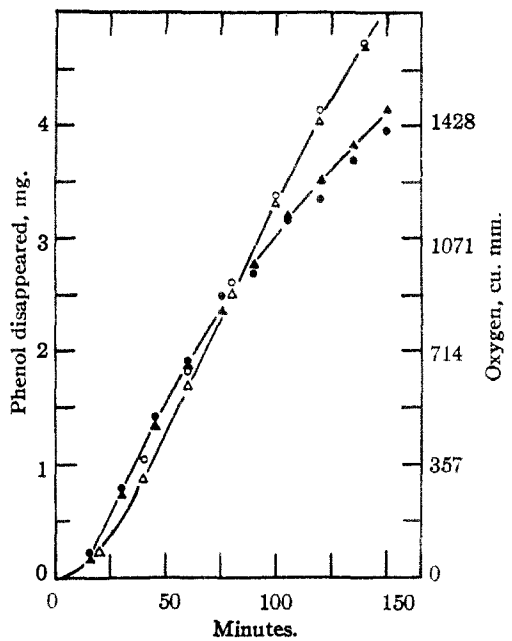


Fig. 1.—Figure showing the rates of disappearance of phenol and of oxygen uptake for the tyrosinase catalyzed aerobic oxidation of phenol at 25°. Warburg respirometer[8] was used in the oxygen uptake measurements. Curve II, triangles, shows the rate of oxygen uptake for a reaction mixture consisting of 2 cc. of *M*/15 phosphate buffer solution (*p*H 6.7), 1 cc. of phenol solution(20 mg.),[9] 1 cc. of gelatin solution (0.2 mg. of gelatin),[11] 1 cc. of a high catecholase preparation, containing 1.5 phenolase, 2.6 cresolase[6] and 32 catecholase units[12] (added at zero time from the side arm of the Warburg reaction flask) and sufficient water to make the final volume in the reaction vessel 8 cc. Curve I, circles, shows the rate of phenol disappearance from a reaction mixture similar to that for Curve II, except that 50 times the quantities of reactants were used. Curve IV, shaded triangles, shows the rate of oxygen uptake for a reaction mixture similar to that for Curve II, except 1 cc. of a high cresolase preparation, containing 1.5 phenolase, 1.3 cresolase, 17 catecholase units was used, and 1 cc. of water replaced the 1 cc. of gelatin solution. Curve III, shaded circles, shows the rate of disappearance of phenol from a reaction mixture similar to that used for the data represented by Curve IV, using, however, 50-fold quantities of the reactants. The reaction vessel used in determining the rate of disappearance of phenol consisted of a modified Reinder-Vles apparatus.[4] Briefly, the latter consisted of a 2-liter glass bottle provided with a rotating glass stirrer, so constructed that air was sucked into the reaction mixture. At suitable intervals 25-cc. samples were withdrawn and the phenol determined. Since both theoretically and experimentally, 1 mg. of phenol requires 357 c. mm. of oxygen for complete oxidation to the 3 atom stage, the data were plotted for purpose of comparison of the rates of phenol disappearance with the rate of oxygen uptake so that 357 c. mm. of oxygen uptake and 1 mg. loss of phenol had the same ordinate value in the graph. The tyrosinase preparations were obtained from the common mushroom, *Psalliota campestris*.

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

(9) To obtain the maximum activity tyrosinase preparations, especially high cresolase, require optimum substrate concentrations.[10] The high catecholase preparation used for Curves I and II had an optimum phenol concentration of 2 mg. of phenol per 8 cc. of reaction mixture, while the high cresolase preparation (Curves III and IV) required about 20 mg. of phenol per 8 cc. Since the use of 20 mg. of phenol per 8 cc. of reaction mixture did not alter to any extent the activity of the high catecholase preparation, this concentration of substrate was used in all experiments.

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

(11) Gelatin was used to stabilize the tyrosinase preparation.[4]

(12) Miller, Mallette, Roth and Dawson, THIS JOURNAL, **66**, 514 (1944).

tioned that in the case of tyrosinase preparations, in which most of the concomitant protein has been removed, the phenolase activity is not very different from the *p*-cresol activity. (For units of activity of the preparations used in the investigation, see the legend for the accompanying figure.) The preparation used in obtaining the results represented by Curves I and II was a high catecholase.

The monohydric phenol activity being dependent on the reaction represented by equation (1) and the *o*-dihydric phenol activity depending on the reactions corresponding to equations (2), (3), and (4), it was decided to compare also the method, based on the rate of phenol disappearance, with that based on the rate of oxygen uptake, when a high cresolase was used instead of a high catecholase preparation. The results obtained are represented by Curves III and IV. It will be observed that again the linear portions of Curves III and IV—the portions of the curves used for determining the units of activity—practically superimpose. Therefore, the method based on the rate of oxygen uptake also is applicable (for high cresolase as well as for high catecholase preparations) for determining the activity toward phenol.

Experimental

The method used in determining the phenol remaining unoxidized in the reaction mixtures, was based on the procedure described by Koppeschar.[13] The phenol was brominated to tribromophenol and the excess bromine determined by potassium iodide and standard sodium thiosulfate solution. A 25-cc. sample of the reaction mixture (see legend for the figure) was added to 10 cc. of 6 *M* acetic acid solution contained in a 250-cc. flask and the mixture shaken for fifteen minutes. (The acid inactivated the enzyme.) After shaking, 3 cc. of a gelatin solution, containing 15 mg. of gelatin, was added and then the solution made alkaline by adding 15 cc. of 6 *M* sodium hydroxide solution.[14] After the mixture had stood for two hours, 10 cc. of a 20% lead acetate solution was added, and the resulting precipitate, consisting of lead phosphate, lead salt of catechol, protein from the enzyme and gelatin, and polymerized hydroxyquinone was filtered off. The filtrate still retained a slight color, due to the hydroxyquinone being acidic and hence somewhat soluble in the alkaline solution.

To remove this remaining trace of soluble hydroxyquinone, 5 cc. of a mixture consisting of equal volumes of *M*/15 potassium dihydrogen phosphate and *M*/15 sodium biphosphate solutions were added, followed by adding consecutively first 2.5 cc. of a 20% lead acetate solution and then 5 cc. more of the phosphate mixture. The precipitate formed was removed by filtering through a Büchner funnel in such a way that the stem of the filter extended to the bottom of the suction flask, to which 20 cc. of 6 *M* sodium hydroxide solution had been previously added. The purpose of this mode of filtering was to prevent any

(13) Koppeschar, *Z. anal. Chem.*, **16**, 233 (1876).

(14) In the alkaline solution, any incomplete oxidation of partly oxidized phenol, such as catechol or *o*-quinone, was converted by autoxidation into the final difficultly soluble polymerized hydroxyquinone. The presence of the gelatin seemed to aid the coagulation, probably by adsorption, of this final oxidation product.

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.

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[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).