Conclusions

1. We have shown that kojic acid reacts with aldehydes to form solid derivatives. A series of these products has been prepared using all of the normal, saturated, aliphatic aldehydes up to heptaldehyde, in addition to acrolein, benzaldehyde, hydrocinnamaldehyde, cinnamaldehyde, and α-furfuraldehyde.

2. The probable formula for these products is given.

3. A possible mechanism of reaction is suggested based upon the present theory of resin formation by aromatic phenols and aldehydes.

MANHATTAN, KAN. RECEIVED MARCH 14, 1938

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

On the Mechanism of the Catechol–Tyrosinase Reaction

BY HARRY WAGREICH AND J. M. NELSON

Catechol has been used widely as a substrate in the study of oxidases, partly because the aerobic oxidation of this substance is catalyzed by a great many enzymes of this class. Robinson and McCance[1] showed that two atoms of oxygen are consumed in the complete oxidation of catechol, although only one atom is required, stoichiometrically, for the formation of *o*-benzoquinone, the normal product of primary oxidation. The reaction therefore is not a simple one, and a further complication arises from the fact that *o*-benzoquinone is very fugitive in aqueous solutions, especially in the presence of unoxidized catechol, as shown by Dawson and Nelson.[2]

Regarding the mechanism of the oxidation, Onslow,[3] Richter,[4] Platt and Wormall[5] and others favor the idea that hydrogen peroxide is one product of the reaction, which would account for the oxygen consumption

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

On the other hand, Raper[6] suggests that one atom of oxygen converts catechol into *o*-benzoquinone and that this is oxidized further by the second atom consumed. To gain further insight into the problem, it seemed of interest to extend the observations of Dawson and Nelson by following the amount of oxygen consumed.

The apparatus shown in Fig. 1 was used to measure the amount of quinone formed when one atom of oxygen per mole of catechol had been consumed. The vessel was charged with 1250 cc. of 0.02 *M* phosphate–citrate buffer (*p*H 6.2) containing 100 mg. of catechol, and 500 units of tyrosinase preparation[7] (about 15 cc.) was placed in one of the side bulbs (F). The stirrer was started and, when the solutions in the vessel and

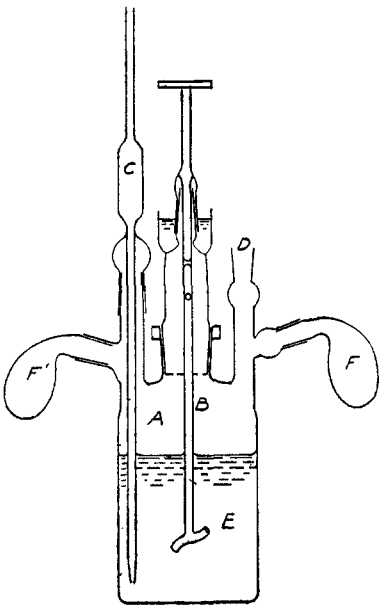


Fig. 1.—A modified form of the apparatus described by Reinders and Vles [*Rec. trav. chim.*, **44**, 1 (1925)]. A represents a heavy 2-liter glass reaction vessel, into which a pump-stirrer B was inserted. At the upper end of the stirrer was a water seal such as described by Rosenthal, Lorch and Hammett [THIS JOURNAL, **59**, 1795 (1937)]. The pipet C permitted the withdrawal of samples from the reaction solution. Tube D was connected to a manometer similar to that described by Reinders and Vles. Bulbs F and F' were used for introducing the enzyme. The vessel A was fitted into a metal frame so as to be held in position in the thermostat.

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

(2) Charles R. Dawson and J. M. Nelson, THIS JOURNAL, **60**, 245 (1938).

(3) M. Wheldale Onslow, "Principles of Plant Biochemistry," University Press, Cambridge, 1931, p. 135.

(4) D. Richter, *Biochem. J.*, **28**, 901 (1934).

(5) B. S. Platt and A. Wormall, *ibid.*, **21**, 29 (1927).

(6) H. S. Raper, *Physiol. Rev.*, **8**, 245 (1928).

(7) Prepared from the common mushroom, *Psalliota campestris*, by the method of Graubard and Nelson, *J. Biol. Chem.*, **112**, 135 (1935).

side bulb had reached the temperature of the thermostat (25°), the bulb F was turned in order to introduce the enzyme into the reaction mixture. After five minutes the oxygen absorbed corresponded to one atom of oxygen per mole of catechol, and at this point 25 cc. of the solution was withdrawn by means of the pipet C and the quinone determined by the method of Dawson and Nelson. The amount of quinone corresponded to 98% of the catechol oxidized. This observation confirms Raper's view that only one atom of oxygen is utilized in the formation of *o*-benzoquinone. The second atom, therefore, must be involved either in the further oxidation of *o*-benzoquinone or in the oxidation of a product of its decomposition.

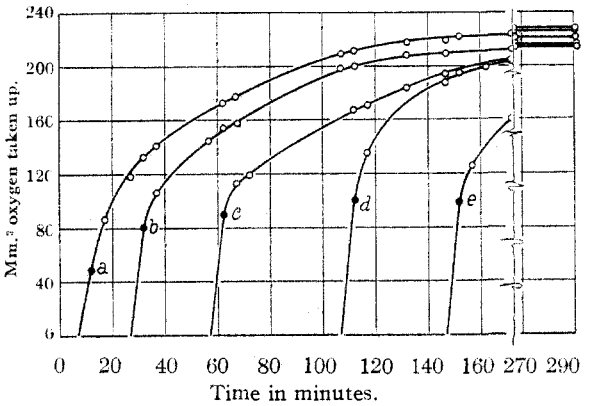


Fig. 2.—Showing the increase in rate of uptake of the second atom of oxygen, with increasing lengths of time elapsing before the enzyme was added to the disappearing *o*-benzoquinone contained in the reaction vessels of the Barcroft-Warburg apparatus. Reaction mixture the same for each curve: 4 cc. of *M* acetate-acetic acid buffer, *p*H 5, 2 cc. catechol solution (2 mg. of catechol); 2 cc. of a solution containing an amount of ceric ammonium sulfate equivalent to oxidizing 2 mg. catechol to *o*-benzoquinone. One cc. of a tyrosinase preparation (3 units) was placed in the side bulb of each reaction vessel, and the enzyme introduced into the reaction mixture at the times indicated on the abscissa axis. Temperature was 25°.

Raper and co-workers[8] found in their excellent study of the enzymatic oxidation of tyrosine to melanin that oxygen was taken up at three different stages in the course of the oxidation, and that the uptake of the last atom of oxygen was independent of the enzyme. To learn whether the uptake of the second atom of oxygen in the oxidation of catechol is catalyzed by tyrosinase the procedure described was followed. To be sure that the first stage was complete, the catechol was first oxidized to *o*-benzoquinone by an equivalent amount of ceric sulfate at *p*H 4.4 (or potassium ferricyanide in the case of more alkaline solutions, *p*H 7.7).[2,9] Since no determination of quinone was necessary, it was more convenient to use the Barcroft-Warburg respirometer method for measuring the amount of oxygen taken up.[10] The results showed that no oxygen uptake occurred in the absence of tyrosinase. When the enzyme was added to the reaction mixture 115 mm.³ of oxygen (1.1 atoms) was consumed at *p*H 4.5 in sixty minutes and at *p*H 7.7 in thirty minutes.

That the substance oxidized by the second atom of oxygen is not *o*-benzoquinone, as proposed by Raper, but some compound formed as the quinone disappears (possibly reformed catechol as discussed more fully in the subsequent part of the paper), is suggested by data shown graphically in Figs. 2 and 3. The data shown in Fig. 2 were obtained by placing a series of aqueous solutions containing a given quantity of catechol, freshly oxidized by an equivalent amount of ceric sulfate to *o*-benzoquinone, in the reaction flasks of a Barcroft-Warburg apparatus. After varying periods of time, as indicated on the abscissa scale in the figure, constant amounts of a tyrosinase preparation were added from the side-arms of the respective reaction flasks containing the gradually changing *o*-benzoquinone. The points marked a, b, c, d, and e on the curves correspond to the amount of oxygen taken up during the first five minutes after the addition of the enzyme. It will be observed upon examining the curves that this amount increased with the length of time elapsing before the enzyme was added (to about one hundred minutes). In other words, as the unstable *o*-benzoquinone disappears a new substance seems to be formed which is oxidized by the tyrosinase. The observed increase in the rate of oxygen uptake is due to the gradual increase in the concentration of this new substance with the length of time elapsing before the addition of the enzyme. The lack of further increase in the rate of oxidation occurring when the length of time elapsing before the addition of the enzyme exceeds one hundred minutes suggests an equilibrium being reached between the disappearing *o*-benzoquinone and the new oxidizable substance. This suggestion falls in line with the observation of Dawson and Nel-

(8) W. C. Evans and H. S. Raper, *Biochem. J.*, **31**, 2162 (1937).

(9) E. G. Ball and T. Chen, *J. Biol. Chem.*, **102**, 691 (1933).

(10) For details of this method, see M. Graubard and J. M. Nelson, *ibid.*, **111**, 757 (1935).

son, who found that *o*-benzoquinone (judging from the liberation of iodine when a portion of the solution was added to acidified potassium iodide solution) did not entirely disappear even after the elapse of considerable time. The same idea is suggested by the curve in Fig. 3 representing the disappearance of *o*-benzoquinone with time, and discussed more fully below.

To obtain, if possible, some idea concerning the quantitative relationship between the amount of *o*-benzoquinone which had disappeared in a given time and the amount of the new oxidizable compound formed, the following experiment was undertaken. A solution consisting of 1030 cc. of water, 200 cc. of *M* sodium acetate–acetic acid buffer (*p*H of final solution 4.8), and 1.1499 g. of ceric ammonium sulfate, was placed in the reaction vessel of the apparatus described in Fig. 1. After the solution had attained the temperature of the thermostat, 100 mg. of catechol dissolved in 25 cc. of water was added, and it was assumed that the catechol was oxidized immediately to *o*-benzoquinone. After thirty-four minutes a 25-cc. portion of the reaction solution was withdrawn and its quinone content was found to have decreased to 10%, based upon the amount of catechol used. To compare this with the amount of the new oxidizable substance formed, 15 cc. of a tyrosinase solution containing 400 units of enzyme was added immediately to the reaction solution after the 25-cc. sample had been withdrawn for determining the amount of quinone remaining at the end of thirty-four minutes. Suspecting the new substance formed as the *o*-benzoquinone disappears to be catechol, which is known to be oxidized rapidly by tyrosinase, 25-cc. portions of the reaction solution were withdrawn one and one-half and three and one-half minutes after the addition of the enzyme. On determining the quinone content it was found to have risen from 10 to 54% at the end of three and one-half minutes. Half of 90%, the loss in quinone at the end of thirty-four minutes, added to the 10% of quinone remaining, is equal to 55%. The experiment was repeated several times, at *p*H 4.5, and similar results were obtained. Thus, interrupting the disappearance of the *o*-benzoquinone, in one instance at 60% and in another at 38% quinone remaining, it was found that on the addition of enzyme the quinone content rose to 80 and 66%, respectively (calcd. 80 and 69%). Another point which should be mentioned is that the newly formed quinone is fugitive just like the original *o*-benzoquinone. The points indicated on the last part of the curve in Fig. 3 represent the quinone content of the reaction solution at the corresponding time intervals.

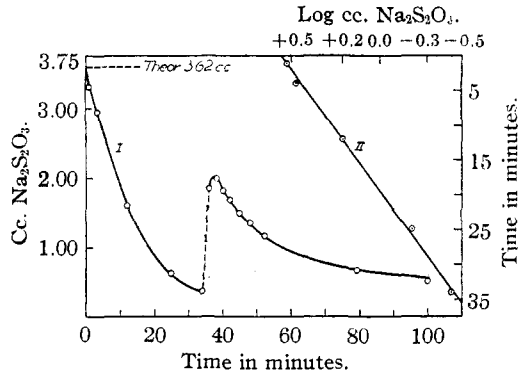
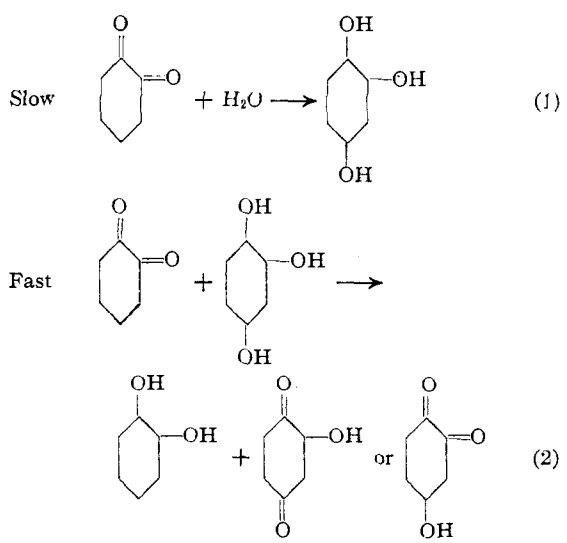
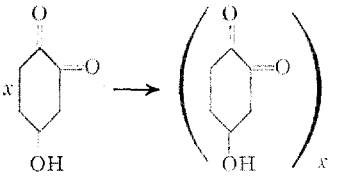


Fig. 3.—Showing the extent of disappearance of *o*-benzoquinone in aqueous solution, and the increase in quinone content, when the product resulting from the disappearing *o*-benzoquinone was oxidized by means of tyrosinase. *p*H = 4.8; temperature 25°; 1 cc. of thiosulfate soln. = 0.539 mg. of *o*-benzoquinone.

In attempting to interpret the data described above, the conclusion reached by Fieser and Peters[11] in their study of β-naphthoquinone is of interest. This quinone also tends to disappear in the presence of water. If the reactions involved in the disappearance of *o*-benzoquinone are similar to those occurring, according to these investigators, in the case of β-naphthoquinone, then they may be expressed in the form of equations (1) and (2)



(11) L. F. Fieser and M. A. Peters, THIS JOURNAL, **53**, 793 (1931).



(3)

For every two molecules of *o*-benzoquinone disappearing, one molecule of catechol would be formed, and this in the presence of the enzyme would yield one new molecule of *o*-benzoquinone. Furthermore, if the reaction represented by equation (1) is slower than the reaction indicated in equation (2), then the rate of disappearance of the quinone should tend to be of the first order. The latter seems to be the case, since plotting the logarithms of the cc. of thiosulfate (measure of the quinone remaining) against time gives a straight line, *i. e.*, curve II in Fig. 3. By adding the assumption represented by equation (3) that the hydroxybenzoquinone disappears from the reaction solution, through polymerization, at a rate greater than that of the reaction represented by equation (1), then results such as those described above should be expected. That hydroxybenzoquinone is unstable, at least in alkaline solutions, has been shown by Eller.[12] who found that alkaline oxidation of hydroquinone or catechol results in the formation of humic acid, a dark brown, sparingly soluble polymer of hydroxybenzoquinone. Experiences in this Laboratory also show that aqueous solutions of *o*-benzoquinone, on standing, give rise to the formation of sparingly soluble material resembling humic acid in appearance. In some instances considerable difficulty was encountered in recognizing the disappearance of the starch–iodine blue color, when the quinone contents of solutions were determined by Dawson and Nelson's method.

(12) W. Eller, *Ber.*, **53**, 1473 (1920).

Szent-Györgyi[13] noticed that the addition of catechol to an aqueous solution of *o*-benzoquinone accelerates the disappearance of the latter. Dawson and Nelson point out that the rate of disappearance of *o*-benzoquinone in dilute aqueous solutions conforms closely to a first order reaction, but deviates from the latter in the presence of higher concentrations of catechol. In the light of these observations, it is possible that the reactions indicated by equations (1) and (2) may only represent the course of the reaction when dilute solutions of catechol are oxidized rapidly by tyrosinase so as to avoid the presence of an appreciable quantity of unoxidized catechol and, under relatively acid conditions, *p*H 4.0–6.0.

Summary

1. Under conditions of low concentration of substrate, high concentration of enzyme, and *p*H 4.5–6.5, catechol is enzymatically oxidized to *o*-benzoquinone with the consumption of one atom of oxygen per mole of catechol.

2. The consumption of the second atom of oxygen per mole of catechol oxidized is also catalyzed by tyrosinase.

3. A substance is formed when *o*-benzoquinone disappears in aqueous solution at *p*H 4.5–6.5, which is aerobically oxidized to a quinone by means of tyrosinase.

4. The quantity of quinone compound formed in the oxidation of the substance mentioned in (3) corresponds to one-half of the *o*-benzoquinone which has disappeared.

5. The substance formed as the *o*-benzoquinone disappears in aqueous solution is oxidized faster by means of the enzyme than the *o*-benzoquinone itself.

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(13) A. von Szent-Györgyi, *Biochem. Z.*, **162**, 399 (1925).