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CHARLESTON, WEST VIRGINIA

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Reaction-Inactivation of Polyphenol Oxidase: Catechol and Oxygen Dependence

BY LLOYD L. INGRAHAM

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Further studies of the reaction-inactivation of polyphenol oxidase have shown that both the reaction-inactivation and enzymatic oxidation of catechol have the same Michaelis–Menten dependence on catechol concentration and oxygen pressure. This is interpreted as meaning that the reaction-inactivation and oxidation mechanism are identical until the enzyme–semiquinone complex is formed. These results are in agreement with the previously proposed idea that the enzyme–semiquinone complex may decompose by either of two pathways: (1) to active enzyme and semiquinone or (2) to inactive enzyme.

During the polyphenol oxidase catalyzed aerobic oxidation of catechol the enzyme activity gradually decreases and finally disappears after about 20 minutes. This effect, called reaction-inactivation,[1] has been shown in previous research to depend upon the first power of the enzyme concentration.[2]

It has been proposed that the reaction-inactivation is due to the possibility that the enzyme–semiquinone complex may decompose by either of two pathways: (1) to active enzyme and semiquinone or (2) to inactive enzyme by an internal oxidation–reduction reaction. The over-all mechanism would be described by the reactions[3]

$$E + O_2 \rightleftharpoons EO_2 \text{ or } E_0 + H_2O$$
$$E_0 + C \rightleftharpoons E_0C$$
$$E_0C \rightleftharpoons ES$$
$$ES \longrightarrow E + S$$
$$ES \longrightarrow \text{inactive enzyme}$$

where E is reduced enzyme, E_0 is oxidized enzyme, C is catechol, S is semiquinone and O_2 is oxygen. If this mechanism holds, the formation of product must have a Michaelis–Menten dependence on not only the catechol concentration but also the oxygen concentration. In addition, however, the reaction-inactivation must also have a Michaelis–Menten dependence on both catechol concentration and oxygen pressure. The integrated rate equation which includes the dependence on catechol concentration and oxygen pressure may be derived in the same manner as equation 1 which is only valid for the experimental conditions where the oxidation is zero order in catechol concentration and oxygen pressure.

$$Q = \frac{k_0'E_0}{k_1'}[1 - e^{-k_1't}] \qquad (1)$$

This equation shows how the amount of oxidation, Q, varies in time, t, at given initial concentration of enzyme, E_0. The "constants" k_0' and k_1' are the rate constants for oxidation and inactivation, respectively, which also include the catechol and oxygen dependence of each reaction. If we substitute $k_0' = k_0f(C,O)$ and $k_1' = k_1g(C,O)$ where $f(C,O)$ is the Michaelis–Menten dependence the oxidation reaction has on catechol and oxygen and $g(C,O)$ is the Michaelis–Menten dependence the inactivation has on catechol and oxygen in the rate equations used to derive equation 1, the integration of the rate equation is not affected. This is because the catechol concentration and oxygen pressure are kept constant during the reaction in our experiments. The catechol concentration is kept constant by adding enough ascorbic acid to the reaction mixture to continually reduce any semiquinones or *o*-quinones formed back to catechol and the oxygen pressure is kept constant by passing a slow stream of an oxygen–nitrogen gas mixture through the reaction mixture. Since the integration is not affected by the substitution one may in effect substitute the values for k_0' and k_1' in equation 1 to obtain equation 2

$$Q = \frac{k_0f(C,O)E_0}{k_1g(C,O)}[1 - e^{-k_1g(C,O)t}] \qquad (2)$$

which holds at all concentrations of catechol and all oxygen pressures. Equation 2 predicts, as one would expect, that the initial rate of reaction

$$\left(\frac{dQ}{dt}\right)_{t=0} = k_0E_0f(C,O) \qquad (3)$$

has a Michaelis–Menten relationship on both catechol concentration and oxygen pressure. However, equation 2 also predicts that if our proposed mechanism is valid, then the ultimate amount of oxidation

$$\lim_{t\to\infty} Q = \frac{k_0f(C,O)E_0}{k_1g(C,O)} = \frac{k_0E_0}{k_1} \qquad (4)$$

is independent of both the catechol concentration and oxygen pressure, since $f(C,O)$ is identical with $g(C,O)$ in the proposed mechanism.

Experimental values of the initial rates of oxidation catalyzed by mushroom enzyme at various percentages of oxygen–nitrogen mixtures are shown in Fig. 1. A locus of the experimental values is found to approximate the theoretical Michaelis–Menten curve corresponding to a maximum activity of 20 and a Michaelis constant of 9% oxygen.

(1) See I. Asimov and C. R. Dawson, THIS JOURNAL, **72**, 820 (1950) and preceding papers.

(2) L. L. Ingraham, J. Corse and B. Makower, *ibid.*, **74**, 2623 (1952).

(3) L. L. Ingraham, *ibid.*, **76**, 3777 (1954).

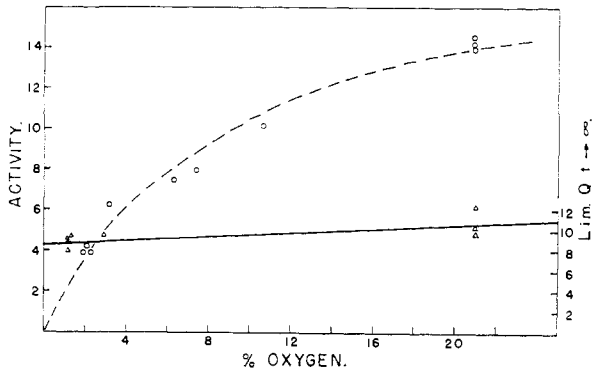

Fig. 1.—The activity in chronometric units per ml. of enzyme and $\lim_{t \to \infty} Q$ in mg. of ascorbic acid per ml. of enzyme *versus* per cent. oxygen in oxygen–nitrogen mixtures. The circles are activities and the triangles are $\lim_{t \to \infty} Q$. The enzyme was mushroom juice. The reaction mixture contained 400 mg. catechol/100 ml. The temperature was 24.0°. The theoretical curve through the circles was calculated for a maximum activity of 20 and a Michaelis constant of 9% oxygen. These values were determined by a Lineweaver–Burk plot of the experimental data. The theoretical curves in Figs. 2 and 3 were also determined in this manner.

Similarly, a locus of the values of the initial rates of oxidation at various catechol concentrations is seen in Fig. 2 to follow the theoretical curve corresponding to a maximum activity of 20 and a Michaelis constant of 0.45 millimolar catechol. With these facts in mind, equation 3 shows that $f(C,O)$ designates a Michaelis–Menten dependence on both catechol concentration and oxygen pressure. The oxygen dependence of the rate of oxidation of a sample of prune enzyme is shown in Fig. 3 for comparison. The difference between the dependence for mushroom and prune enzyme is probably within experimental error.

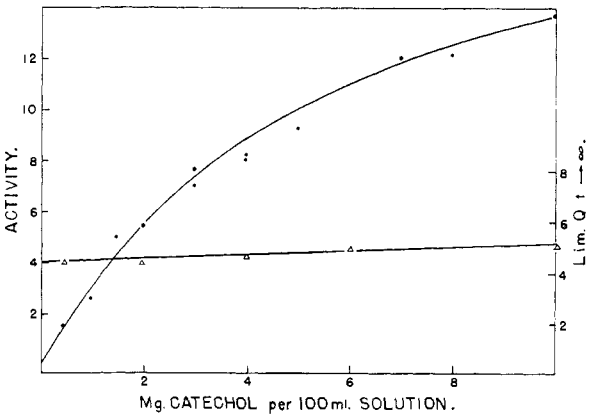

Fig. 2.—Activity of commercial mushroom enzyme in chronometric units per ml. and $\lim_{t \to \infty} Q$ in mg. ascorbic acid per ml. of enzyme *versus* catechol concentration in mg. catechol per 100 ml. reaction mixture. The circles are activity measurements and the triangles are $\lim_{t \to \infty} Q$ measurements. The curve through the circles corresponds to a maximum activity of 20 and a Michaelis constant of 5 mg. catechol per 100 ml. or 0.45 millimolar. The activity measurements were made on a solution containing 3.7 mg. enzyme per ml. and the $\lim_{t \to \infty} Q$ measurements on a solution containing 2.5 mg. enzyme per ml.; temperature 25.0°.

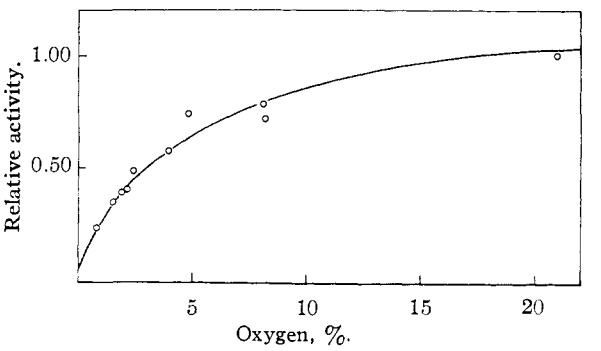

Fig. 3.—Oxygen dependence of a preparation of enzyme from prunes containing about 100 chronometric units/ml. at 20.9% oxygen. The enzyme decreased in activity during the course of the investigation so that all activities are referred to a relative activity of 1.00 at 20.9% oxygen; measurements at 24.0°. Reaction mixture contained 500 mg. catechol per 100 ml. Curve corresponds to a maximum activity of 1.27 and a Michaelis constant of 5.0% oxygen.

The results of studies of the ultimate amount of oxidation, as a function of oxygen pressure and catechol concentration are also shown in Figs. 1 and 2. It is important to notice that over the same range of both oxygen pressures and catechol concentrations where the initial rate of oxidation was found to have a Michaelis–Menten dependence on catechol and oxygen, $\lim_{t \to \infty} Q$ is independent of oxygen pressure and catechol concentrations. This is in agreement with the predictions of our proposed mechanism for reaction-inactivation.

The prune preparation gave so little reaction-inactivation that $\lim_{t \to \infty} Q$ could not be quantitatively measured.

Experimental

The initial rates of oxidation were measured by the chronometric method[4] using the potentiometric end-point.[5] Lim Q was measured colorimetrically as described in a previous paper.[3]

The low pressures of oxygen were obtained by a flow system. The gases (oxygen and nitrogen) were supplied by cylinders through double stage reducing valves to five-gallon ballast tanks. The gases in the ballast tanks at approximately 5 lb./sq. inch were controlled by two needle valves through two Rotameter flowmeters. From the readings on the flowmeters were calculated the values given in Fig. 2. The gases were then mixed and passed into a one-gallon surge tank. From here by means of needle values a portion of the approximately 2 liters/min. flow was bubbled through the reaction cell and the rest was allowed to escape into the air. The high flow rate was needed in order to use the most accurate portion of the flow meter scale. The gas was bubbled through the reaction solution for ten minutes before the reaction was initiated by the addition of enzyme.

Enzyme.—The commercial mushroom enzyme was obtained from Mann Biochemical Company. The mushroom juice enzyme has been described in a previous investigation.[3] The prune enzyme was prepared essentially as previously described for apple enzyme.[6]

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(4) W. H. Miller, M. F. Mallette, L. J. Roth and C. R. Dawson, THIS JOURNAL, **66**, 514 (1944).

(5) L. L. Ingraham and B. Makower, to be submitted for publication soon.

(6) J. D. Ponting and M. A. Joslyn, *Arch. Biochem.*, **19**, 47 (1948).