ously extracted with ether. Evaporation of the ether from the first ether extraction gave scopolamine (4.2 g.) as a cloudy viscous oil.

Evaporation of the ether from the continuous extraction, after drying over anhydrous magnesium sulfate, gave 3.35 g. of scopine as a clear viscous oil. This is a 96% yield based on unrecovered scopolamine.

The colorless oil was taken up in excess pentane and treated with 0.1 g. of charcoal. After removal of the charcoal, hexane was added and the solution was concentrated by gentle warming. The solution was cooled to room temperature and then in the refrigerator overnight. This gave crystalline scopine, m.p. 73–75°. One further crystallization from pentane gave colorless needles, m.p. 76°.

Solid scopine as a Nujol mull showed the following infrared maxima: 3.04, 7.52, 7.65, 7.80, 8.07, 8.23, 8.33, 8.68, 8.85, 9.28, 9.60, 9.77, 10.05, 10.18, 10.60, 11.50, 11.80, 12.29, 13.70 and 14.11 μ.

Scopine in aqueous solution formed a picrate in good yield when treated with a methanolic solution of picric acid. The picrate had m.p. 242–243° (identical with the melting point of authentic scopoline picrate). A mixture melting point with authentic scopoline picrate showed no depression. The infrared spectra of scopine picrate and scopoline picrate were, however, distinctly different, each spectrum containing maxima totally absent in the other. The conversion of scopine picrate to scopoline picrate on heating to the melting point is not surprising in view of the report by Moffett and Garrett that scopine methobromide rearranges to scopoline methobromide on heating to the melting point.[5]

Scopine Methobromide.—A solution of scopine in methanol was treated with excess methyl bromide and left overnight at 0°. Scopine methobromide was then collected in nearly quantitative yield (m.p. 294–296° dec.).

Scopoline (III).—Scopolamine, dissolved in excess 10% potassium hydroxide, gave scopoline as a clear viscous oil in 98% yield after 17 hours reflux.

Scopoline showed the following infrared maxima: 2.97, 3.47, 6.86, 6.98, 7.16, 7.41, 7.54, 7.65, 7.74, 8.04, 8.21, 8.35, 8.56, 8.95, 9.31, 9.49, 9.60, 9.68, 9.82, 9.98, 10.20, 11.08, 11.20, 11.44, 12.00, 12.21, 12.50, 12.95 and 14.30 μ.

Scopoline picrate, obtained by mixing an aqueous solution of picric acid an an aqueous solution of scopoline, had m.p. 242–243° dec.

ITHACA, NEW YORK

[CONTRIBUTION FROM THE WESTERN UTILIZATION RESEARCH BRANCH, AGRICULTURAL RESEARCH SERVICE, UNITED STATES DEPARTMENT OF AGRICULTURE]

Variation of the Michaelis Constant in Polyphenol Oxidase Catalyzed Oxidations: Substrate Structure and Concentration

BY LLOYD L. INGRAHAM

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A study is reported of the variation of the oxygen Michaelis constant with the structure of the hydrogen donor in a system catalyzed by polyphenol oxidase. Three hydrogen donors, catechol, chlorogenic acid and caffeic acid, were chosen for this study. From the assumption that the rate constants governing the equilibrium between enzyme and oxygen are independent of hydrogen donor structure and the fact that the Michaelis constant for oxygen is highly dependent upon the maximum velocity for three different hydrogen donors, the conclusion is drawn that the oxygen Michaelis constant is not an equilibrium constant. A study is also reported of the variation of the oxygen Michaelis constant with the hydrogen donor concentration. This effect had been predicted previously from theoretical considerations. The Michaelis constants approach a value of ~1.5% oxygen at low values of the hydrogen donor concentration regardless of the hydrogen donor structure. These facts are consistent with the ideas that the equilibrium constant between enzyme and oxygen is ~1.5% oxygen and also that the enzyme combines with oxygen before it does with the hydrogen donor.

In a recent paper[1] the kinetics for a two-substrate enzyme-catalyzed system were discussed. This discussion showed theoretically how the Michaelis constant for one substrate may vary with the concentration of the other substrate. Previous variations of Michaelis constants have been reported for glucose oxidase,[2] lipoxidase[3] and dextransucrase.[4] The purpose of this paper is to report a study of the variation of the Michaelis constant for oxygen with both structure and concentration of the other substrates, the hydrogen donors, in the polyphenol oxidase catalyzed system and also a discussion of the significance of these variations in understanding the mechanism of the polyphenol oxidase catalyzed aerobic oxidation of catechol.

Measurements of Michaelis Constants.—The Michaelis constants for the substrate oxygen were determined from the curvature in the plots of the consumption of oxygen *vs.* time as measured by means of a rotating polarized electrode.[5] The decrease in rate of oxygen consumption with time may be the result of either or both the diminution of the oxygen supply in solution or a decrease in catalytic activity of the enzyme from reaction-inactivation.[6,7] The hydrogen donor is kept at a constant concentration by the excess of ascorbic acid in solution. One may calculate when reaction-inactivation is important in affecting the curvature as follows:

The rate of consumption of oxygen has a Michaelis dependence on oxygen concentration[8]

$$\frac{d(O_2)}{dt} = -\frac{k_0(E)(O_2)}{(O)_2 + K_m} \qquad I$$

where k_0 is the rate constant for oxidation, K_m is the Michaelis constant and (E) is the enzyme concentration. In addition, the rate of disappearance of enzyme

$$\frac{d(E)}{dt} = -\frac{k_1(E)(O_2)}{(O)_2 + K} \qquad II$$

has a similar dependence on oxygen concentration.[8] A combination of equations I and II gives

$$k_1\, d(O_2) = k_0 d(E) \qquad III$$

(1) L. L. Ingraham and B. Makower, *J. Phys. Chem.*, **58**, 266 (1954); see also J. Z. Hearon, *Physiol. Revs.*, **32**, 499 (1952).

(2) H. Laser, *Proc. Roy. Soc. (London)*, **140B**, 230 (1952).

(3) A. L. Tappel, P. D. Boyer and W. O. Lundberg, *J. Biol. Chem.*, **199**, 267 (1952).

(4) H. M. Tsuchiya and C. S. Stringer, Soc. Am. Bact. Meeting, New York, N. Y., 1955.

(5) L. L. Ingraham, *Anal. Chem.*, **28**, 1177 (1956).

(6) W. H. Miller and C. R. Dawson, THIS JOURNAL, **63**, 3375 (1941).

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

(8) L. L. Ingraham, *ibid.*, **77** 2875 (1955).

Equation III may be integrated and the value of (E) substituted in equation I. The resulting equation may be integrated to give equation IV.

$$\frac{a}{V_0}\frac{(a - S_0 - K)}{(a - S_0)}\ln\frac{(a + S - S_0)}{(a)} + \frac{K_m}{V_0}\frac{(a)}{(a - S_0)}\ln\frac{S}{S_0} = -t \quad \text{IV}$$

In the above equation, the ultimate amount of ascorbic acid oxidized $a = k_0E_0/k_1$ and the initial velocity, $V_0 = k_0E_0$. The symbols E_0 and S_0 denote initial enzyme and oxygen concentrations, respectively, and S denotes the oxygen concentration at time, t. When $S_0 + K \ll a$ then equation IV reduces to equation V

$$S - S_0 + K_m \ln\frac{S}{S_0} = -V_0t \quad \text{V}$$

the integrated Michaelis equation for one substrate. Therefore, when $S_0 + K \ll a$, which is commonly true, the experimental curves drawn by the recording potentiometer obey equation V. Curves are shown in Fig. 1 for two substrates having a large difference in oxygen dependence. The easiest way to determine the values for V_0 and K is to read the rates directly from these curves by means of a prism and to construct conventional Lineweaver–Burk plots. The parameters found from these plots have been used in the integrated equation V to calculate the points shown in Fig. 1.

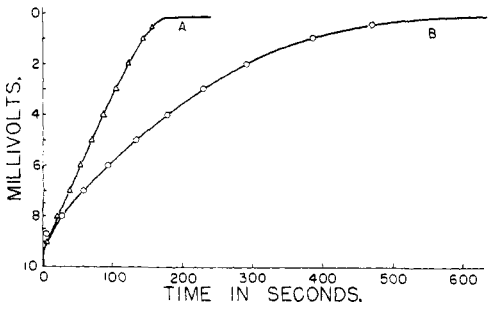

Fig. 1.—A recorder tracing of the oxygen consumption of caffeic acid (A) superimposed over a similar tracing for chlorogenic acid (B). The vertical axis measures oxygen (expressed as millivolts) and the horizontal axis measures time. Notice how the caffeic acid rate is almost constant to quite low oxygen concentrations whereas the chlorogenic acid rate decreases very rapidly with a decrease in oxygen. The effect is even more pronounced than these curves indicate because the caffeic acid solution was saturated with air and the chlorogenic acid solution was saturated with oxygen. The recorder range was changed between determinations so that both curves would use the full width of the paper. The circles were calculated from equation V with K_m = 42% oxygen and V_0 = 0.0420 mv./second. The triangles were calculated with K_m = 1.1% oxygen and V_0 = 0.0675 mv./second.

Notice all the points fit the experimental curves quite well except one at the base of one curve. This was a common occurrence and is probably because the electrode or cell required a few seconds to come to equilibrium. For this reason the value of initial time was in doubt and it was easier to construct Lineweaver–Burk plots than plots of equation V which requires a knowledge of the initial time. However, in all determinations the parameters determined by the Lineweaver–Burk plots fit the integrated curves quite well.

Experimental

The enzyme used in these determinations was prepared from French prunes by the same procedure as used by Baruah and Swain[9] to prepare potato enzyme. All rates were determined at 25°. The solution was 0.02 M in citric acid, 0.04 M in disodium phosphate and 2–3 mM in ascorbic acid. The pH was 5.5. The substrate concentrations are shown in Fig. 2. The Michaelis constants for caffeic acid were determined in solutions saturated with air whereas it was necessary to determine the Michaelis constants of catechol and chlorogenic acid in solutions saturated with oxygen because of higher Michaelis constants for oxygen of these two hydrogen donors. In all determinations the value of a, determined by a method described previously,[10] was found to be at least $10(S_0 + K)$. However, in order to maintain the relationship $a = 10(S_0 + K)$ when determining the Michaelis for oxygen with catechol in pure oxygen it was only possible to analyze the last half of the curve, thus reducing the value of S_0.

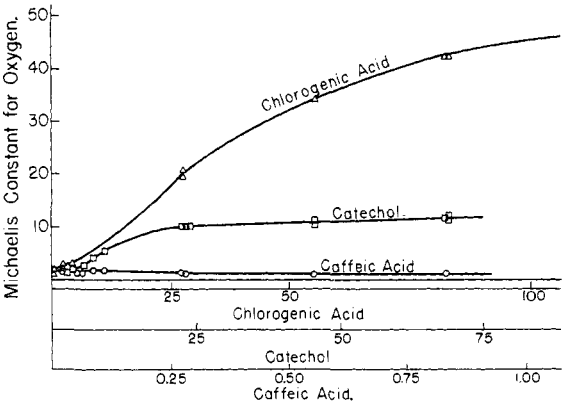

Fig. 2.—The Michaelis constant at 25° for oxygen at various concentrations of 3 hydrogen donors. The electron-donor concentrations are in millimoles per liter. Notice that the oxygen Michaelis constant for the enzyme saturated with chlorogenic acid is very likely higher than that measured at 82 mM chlorogenic acid. A value of 46% oxygen has been estimated. Measurements at higher concentrations were impossible because of the limited solubility of chlorogenic acid.

The values of the Michaelis constants for oxygen are shown in Fig. 2 as a function of the concentrations of the hydrogen donors, catechol, caffeic acid and chlorogenic acid. The Michaelis constants for oxygen are different for the three hydrogen donors at high concentrations of the hydrogen donor but decrease with decreasing concentration of the donors catechol and chlorogenic acid and increase slightly with decreasing concentration of the donor caffeic acid. In all previously reported examples[2-4] the Michaelis constants decreased with decreasing concentration of other substrate. The most noticeable and certainly the most significant result is that all the Michaelis constants approach the same value of approximately 1.5% oxygen at low concentrations of the hydrogen donors for all three donors—catechol, chlorogenic acid and caffeic acid.

The variations of the Michaelis constant for oxygen with hydrogen donor structure and concentration will be discussed separately.

Part I: Hydrogen Donor Structure. Kinetic Form of the Michaelis Constant.—The variation of the Michaelis constant for oxygen on the structure of the hydrogen donor at large concentrations of hydrogen donors enables one to make certain state-

(9) P. Baruah and T. Swain, *Biochem. J.*, **55**, 392 (1953).
(10) L. L. Ingraham, THIS JOURNAL, **76**, 3777 (1954).

ments about the kinetic form of the Michaelis constant for oxygen.

The problem of the kinetic form of the Michaelis constant has long been of interest to enzyme kineticists. Originally Michaelis assumed it was the equilibrium constant for the reaction between enzyme and substrate, but later Briggs and Haldane[11] showed it could be of the form $(k_4 + k_5)/k_3$ where k_3, k_4 and k_5 refer to the reactions

$$S + E \underset{k_4}{\overset{k_3}{\rightleftarrows}} ES \xrightarrow{k_5} E + P$$

In the above reactions, S stands for substrate concentration, E for enzyme concentration, and P for products.

The kinetic forms of Michaelis constants have been determined by a study of the kinetics of the enzyme–substrate complex[12] but this method requires an analytical method for the actual concentration of the enzyme-substrate complex.

An attempt was made to determine the kinetic form of the Michaelis constant for the combination of trypsin with benzoyl-L-argininamide.[13] The Michaelis constant for a substrate slow to hydrolyze was compared with the competitive inhibition constant for this same substrate in the presence of a substrate that hydrolyzes much faster. Because the inhibition constant is usually of the form k_2/k_1 and the Michaelis constant was found experimentally to be numerically equal to the inhibitor constant, it was assumed that the Michaelis constant was of the form k_2/k_1 also. In this system, as we shall prove below, the inhibition constant is not of the form k_2/k_1 but is of the form $(k_2 + k_3)k_1$. We shall assume that $k_3 \gg k'_3$ and that these reactions take place[14]

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_3} E + P$$

$$E + S' \underset{k'_2}{\overset{k'_1}{\rightleftarrows}} ES' \xrightarrow{k'_3} E + P'$$

A solution of the steady state equations for this system gives equation VI

$$\frac{k_3 E_0}{V_I} = 1 + \frac{k_2 + k_3}{k_1}\Big/S + \frac{k_2 + k_3}{k_1}S'\Big/\frac{k'_2 + k'_3}{k'_1} \quad \text{VI}$$

where V_I is the initial rate with the inhibitor S′ present. Comparison of this with the equation for competitive inhibition

$$\frac{k_3 E_0}{V_I} = 1 + \frac{K_m}{S} + \frac{K_m S'}{K'_I S}$$

where K'_I is the inhibition constant, shows that

$$K'_I = \frac{k'_2 + k'_3}{k'_1}$$

As the Michaelis constant, K'_m, is also $(k'_2 + k'_3)/k'_1$, it is clear that no statements can be made about the relative magnitudes of k'_1, k'_2 and k'_3 if K'_m is found to be equal numerically to K'_I.

In the method described in this paper to determine the kinetic form of the Michaelis constant, advantage is taken of the dependence of the Michaelis constant on hydrogen donor structure at high concentration of the hydrogen donors. One may assume that k_4 and k_5, the rate constants governing the equilibrium between enzyme and oxygen, are independent of hydrogen donor structure. However, the rate constant, k_5, determining the rate of formation of product is certainly dependent upon hydrogen donor structure. A plot of the oxygen Michaelis constant against k_5 for hydrogen donors of different structures would indicate the kinetic form of the Michaelis constant. The form $(k_4 + k_5)/k_3$ would give a graph with a slope of $1/k_4$ and an intercept on the $(k_4 + k_5)/k_3$ axis of k_4/k_3. If the Michaelis constant is of the form k_4/k_3 the Michaelis constant for oxygen would be independent of the hydrogen donor structure. If the Michaelis constant is of the form k_5/k_3, it would be proportional to the k_5 for the various hydrogen donors. It should be pointed out that this method of determining the kinetic form of the Michaelis constant is quite similar to the ideas presented by Lumry, Smith and Glantz.[15]

Actually the kinetics for this two-substrate system, oxygen and hydrogen donor, are more complex than indicated above. The assumption that the enzyme combines with one of the substrates faster than the other one and the fact that the rates have a first-order dependence on substrate at low concentrations of both substrates[8] but a zero-order dependence on substrate at high concentrations of both substrates[8] leads to the reaction mechanism

$$A + E \underset{k_2}{\overset{k_1}{\rightleftarrows}} EA$$

$$B + EA \underset{k_4}{\overset{k_3}{\rightleftarrows}} EAB$$

$$EAB \xrightarrow{k_5} E + P$$

The symbols A and B stand for the two substrates, E stands for enzyme, and P for product. The Michaelis constants for this system[1] are summarized in Table I. *A priori*, it is not possible to know

TABLE I

MICHAELIS CONSTANTS

Concn. of B	For A
Any	$\frac{k_2k_4 + k_2k_5 + k_3k_5B}{k_1(k_4 + k_5 + k_3B)}$
High	k_5/k_1
Low	k_2/k_1
Concn. of A	**For B**
Any	$\frac{k_2k_4 + k_1k_4A + k_2k_5 + k_1k_5A}{k_3k_5 + k_1k_3A}$
High	$(k_4 + k_5)/k_3$
Low	$\left(\frac{k_2}{k_5}\right)\left(\frac{k_4 + k_5}{k_3}\right)$

(11) G. E. Briggs and J. B. S. Haldane, *Biochem. J.*, **19**, 338 (1925).

(12) B. Chance, D. S. Greenstein and F. J. W. Roughton, *Arch. Biochem. and Biophys.*, **37**, 301 (1952).

(13) S. A. Bernhard, THIS JOURNAL, **77**, 1973 (1955).

(14) These reactions assume that the substrate S′ acts as an inhibitor at the same site on the enzyme as it acts as a substrate. Without this assumption no conclusion whatsoever can be drawn concerning the relative values of the Michaelis constant and the inhibitor constant.

(15) R. Lumry, E. L. Smith and R. R. Glantz, THIS JOURNAL, **73**, 4330 (1951).

which Michaelis constants apply because it is not known which substrate combines with the enzyme first.

At high concentrations of hydrogen donors the Michaelis constants for oxygen may be either k_5/k_1 or $(k_4 + k_5)/k_3$.

Results

In Fig. 3 the Michaelis constants for oxygen are shown as a function of the maximal velocities for these three compounds. It should be pointed out that these values are maximal velocities with respect to both oxygen pressure and hydrogen donor concentrations. The very small intercept indicates either that the Michaelis constant is of the form k_5/k_1 or of the form $(k_4 + k_5)/k_3$ with $k_5 \gg k_4$.

If an error of $\pm 10\%$ is allowed in the Michaelis constants and an error of $\pm 5\%$ in the maximal velocities, the intercept may be as large as ± 0.35 so that the Michaelis constant, K_m, follows the equation VII.

$$K_m = \frac{k_4E_0}{k_3} + \frac{k_5E_0}{k_3} = \pm 0.35 + 10k_5E \qquad \text{VII}$$

If the Michaelis constant is of the form $(k_4 + k_5)/k_3$ and if the outer limit for the intercept is used, the minimum ratios of $k_5/k_4 = 130$ for chlorogenic acid, 31 for catechol and 3.1 for caffeic acid can be derived.

Part II: Hydrogen Donor Concentration.—Although the Michaelis constants for oxygen are quite different for the three hydrogen donors at high concentrations they approach the same value of 1.5% oxygen at low concentrations of each hydrogen donor. This phenomenon will be discussed with the aid of Table I and with two hypotheses as starting points for the discussion.

Hypothesis I. The Enzyme Combines with the Electron Donor First.—Oxygen is B in the reaction sequence and the Michaelis constant for oxygen at high concentrations of the hydrogen donor is $(k_4 + k_5)/k_3$. At low concentrations of the hydrogen donors the Michaelis constant would approach $(k_2/k_5)(k_4 + k_5)/k_3$ or k_2/k_3 if k_4 is neglected with respect to k_5. (This is a good approximation for catechol and chlorogenic acid but rather poor for caffeic acid; *cf.* minimum ratios of k_5/k_4 in part I). One would expect that k_2 would depend upon the hydrogen donor structure. Hypothesis I therefore seems unlikely because the Michaelis constants for oxygen at low concentrations of hydrogen donors were found to be the same for the three different hydrogen donors.

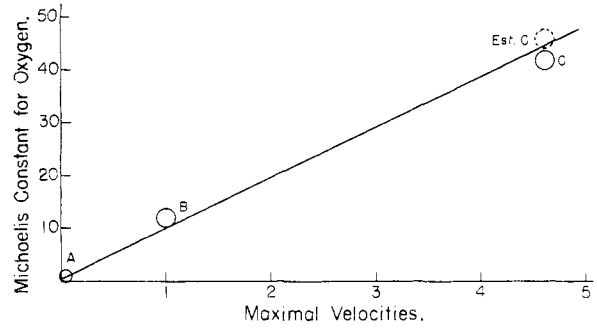

Fig. 3.—The Michaelis constants for oxygen as a function of the maximal velocities of the hydrogen donors, caffeic acid (A), catechol (B) and chlorogenic acid (C). The estimated value for C is described in the legend for Fig. 2.

Hypothesis II. The Enzyme Combines First with Oxygen.—The symbol A is now oxygen and B denotes the hydrogen donor. The Michaelis constant for oxygen at high concentrations of the hydrogen donors is k_5/k_1 which is consistent with the fact that the Michaelis constant for oxygen could be of the form k_5/k_1 (*cf.* discussion, part I). At low concentrations of the hydrogen donors the Michaelis constant for oxygen approaches the value k_2/k_1, the equilibrium constant for the combination of enzyme with oxygen. It seems logical to assume, as was done previously, that this value would be independent of the hydrogen donor structure. The approximate value 1.5% oxygen is therefore a measure of the equilibrium constant between oxygen and enzyme, whereas the values found at higher concentrations of the hydrogen donors are the steady-state Michaelis constants. It is interesting to note that the steady-state Michaelis constant may be either higher (catechol and chlorogenic acid) or lower (caffeic acid) than the equilibrium constant between enzyme and oxygen.

The possibility that the enzyme combined first with the hydrogen donor at high concentrations of hydrogen donor but combined with oxygen first at lower concentrations of hydrogen donors can be ruled out for the hydrogen donors chlorogenic acid and catechol because the oxygen concentration at the Michaelis constant is reduced by about the same factor or more than the hydrogen donor concentration. This possibility exists for the hydrogen donor, caffeic acid.

ALBANY 10, CALIF.