amylose a chain composed entirely of polyanhydroglucuronide groups which are substantially as long as the original starch molecules, if indeed there are perfectly linear molecules in starch. It follows from our work that catalysts or adjuncts other than water or moisture, are required which will promote the reaction and that water should be eliminated as soon as it is formed in the oxidation reaction. Otherwise correction factors are required in the determination of uronic acid carboxyls which appear to be relatively large in respect to the final value which is of importance in starch structure studies and one of the serious disadvantages inherent in other chemical approaches, such as methylation, would not have been overcome.

The assistance given by Mr. O. R. Trubell in performing several of the analyses reported in this communication is gratefully acknowledged.

Summary

A study was made of the reaction between nitrogen dioxide in the gaseous state and corn starch and its fractions in the solid state. The percentage of uronic acid carboxyl groups found in amylose after oxidation was greater than in oxidized amylopectin; whole starch gave a value intermediate to the two fractions.

These results qualitatively support the view that branching through glucoside linkages to carbons number 6 is more extensive in amylopectin than in amylose molecules.

It was found also that the physical form of the carbohydrates, as well as moisture associated with, or formed in the starch, influenced the oxidation reaction rate.

Additional data were given to show that moisture also facilitates hydrolysis of glucoside linkages which makes possible the formation of saccharic acid groups at the points of scission. Carbon-6 carboxyl groups in saccharic acid were found to be relatively more stable in a decarboxylation reaction with hydrochloric acid than uronic acid carboxyls.

ARGO, ILLINOIS RECEIVED MAY 20, 1949

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

On the Reaction Inactivation of Tyrosinase during the Aerobic Oxidation of Catechol[1]

BY ISAAC ASIMOV AND CHARLES R. DAWSON

Tyrosinase is a copper-bearing phenolase widely spread throughout the plant kingdom, notably in the common mushroom, *Psalliota campestris*. This enzyme possesses the property of catalyzing the aerobic oxidation of both monohydric and *o*-dihydric phenols.[2] One of the most striking characteristics of the aerobic oxidation of catechol as catalyzed by the enzyme tyrosinase, is the marked inactivation of the enzyme that occurs during the early course of the reaction. The enzyme inactivation appears to be an integral part of the mechanism whereby tyrosinase catalyzes the oxidation, since the loss of activity, which is markedly in evidence even during the first minute of the reaction,[4] is not brought about by any known product of the enzyme reaction, nor by catechol in an anaerobic system.[5,6] Furthermore, the inactivation during the early course of the reaction cannot be attributed to an instability of the enzyme in dilute solution, or to salt, heat or *p*H effects, since it has been found by the authors that control systems with only catechol lacking showed no loss in enzyme activity over comparable periods in the absence of agitation.[7]

By means of chronometric measurements, Miller, *et al.*,[8] showed experimentally that when the amount of quinone (Q) formed during the tyrosinase-catalyzed oxidation of catechol, was plotted reciprocally against the time of reaction (t), (*i. e.*, $1/Q$ *vs.* $1/t$), a linear relationship was obtained

$$\frac{1}{Q} = \left[\frac{b}{a}\right]\left[\frac{1}{t}\right] + \frac{1}{a} \qquad (1)$$

This relationship may also be expressed

$$Q = at/(b + t) \qquad (2)$$

in which form it has been termed in these laboratories, the "chronometric equation." (The terms, a and b, in these equations are constants, the dimensions of which are equivalent to those used for Q and t, respectively, and which vary in value with

(1) From a dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science of Columbia University by Isaac Asimov, present address: Department of Biochemistry, Boston University School of Medicine.

(2) Since *p*-cresol and catechol are commonly used as experimental substrates these catalytic activities are termed cresolase and catecholase activities, respectively.[3]

(3) J. M. Nelson and C. R. Dawson, *Advances in Enzymology*, **4**, 99 (1944).

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

(5) C. R. Dawson and B. J. Ludwig, *ibid.*, **60**, 1617 (1938).

(6) B. J. Ludwig and J. M. Nelson, *ibid.*, **61**, 2601 (1939).

(7) When such control systems (lacking in catechol) are subjected to agitation, a loss in enzyme activity does result, presumably because of protein denaturation at the air-liquid interface. However, such surface denaturation does not appear to contribute to the inactivation of the enzyme observed during the first three minutes of the aerobic oxidation of catechol in agitated systems ("reaction inactivation") since the same rate and extent of inactivation is observed in unagitated systems.

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

the particular enzyme–substrate system under consideration.)

It is the purpose of this communication: (1) To describe an investigation of the precision with which Equation 2 represents the actual course of quinone formation with time under the conditions of the chronometric technique; (2) to analyze the kinetic implications of Equation 2; and (3) to present an enzyme model which will account for the experimental observations with regard to the reaction inactivation of tyrosinase.

Experimental Procedures and Results

Enzyme.—The enzyme employed in the experiments described below was tyrosinase preparation C259A2, prepared from the common mushroom, *Psalliota campestris*, according to the method described by Mallette, *et al.*[9] The dry weight of the preparation, as determined spectrophotometrically[10] was 0.93 mg. per ml. and the copper content was 0.97 gammas per mg. dry weight (0.097%) as determined by the cysteine-oxidation method.[11,12] The enzyme had a catecholase activity[8] of 2020 units per mg. and a cresolase activity[13,14] of 50.8 units per mg. (catecholase/cresolase ratio, 39.6.[15])

A constant amount of enzyme was employed in all of the experiments described below. It was found that a dilution of the above enzyme solution, so prepared as to contain 155 gammas dry weight per ml. was stable at ice-bath temperatures for at least ten hours. A 0.1-ml. sample of such a dilution was used in each of the chronometric determinations.

The Chronometric Method.—All chronometric experiments were conducted under the same conditions as described by Miller, *et al.*,[8] except for the following modifications in technique: (1) The reaction flask was equipped with ground-glass connections to minimize danger of contamination of the reaction mixture; and a small air-vent was included in the flask, the stopping of which would serve to start the siphon action. (2) To the line conducting the air-stream used to aerate and agitate the reaction mixture, was attached a mercury safety-valve, designed in the form of an open-end manometer. By measuring the differences in mercury levels in the U-tube, it was found possible to reproduce the conditions of aeration and agitation in the reaction flask. Throughout this communication, the term "aeration pressure" (expressed in cm. of mercury) is used to represent this difference in mercury levels. (3) Copper-free water (*i. e.*, laboratory-distilled water, redistilled in an all-Pyrex apparatus) was used in making up all solutions used for the enzyme reaction mixture, for it was observed that traces of copper affected the chronometric end-point by several per cent. through its effect on the autoxidation of ascorbic acid.[16,17] (4) The air-source was not connected to the reaction flask earlier than two or three seconds before the initiation of the reaction through the addition of catechol, since it was observed that the enzyme, in the absence of substrate, was sensitive to surface agitation, losing 5–10% of its activity when exposed to gentle bubbling for as little as thirty seconds.[7]

(9) M. F. Mallette, S. Lewis, S. R. Ames, J. M. Nelson and C. R. Dawson, *Arch. Biochem.*, **16**, 283 (1948).

(10) I. Z. Eiger and C. R. Dawson, *ibid.*, **21**, 181 (1949).

(11) O. Warburg, *Biochem. Z.*, **187**, 255 (1927).

(12) P. L. Lovet-Janison and J. M. Nelson, THIS JOURNAL, **62**, 1409 (1940).

(13) D. C. Gregg and J. M. Nelson, *ibid.*, **62**, 2500 (1940).

(14) M. F. Mallette and C. R. Dawson, *ibid.*, **69**, 466 (1947).

(15) The ratio of catecholase to cresolase activities in a given tyrosinase preparation is dependent on the method used for purification.[9] Where the ratio is relatively high (greater than 20), the enzyme is termed a high catecholase preparation and where relatively low, a high cresolase preparation.

(16) C. M. Lyman, M. O. Schulze and C. G. King, *J. Biol. Chem.*, **118**, 757 (1937).

(17) H. G. Steinman and C. R. Dawson, *ibid.*, **64**, 1212 (1942).

Table I shows the experimental results obtained in a typical chronometric experiment, conducted in the manner described above, using a constant aeration pressure of 2.54 cm.

TABLE I

CHRONOMETRIC EXPERIMENT ($Q - t$ VALUES) AT 2.54 CM. AERATION PRESSURE

Q[a] (mg. ascorbic acid used)	$1/Q$	t[b] (seconds)	$1/t \times 10^3$
1.00	1.000	19.7	50.8
1.50	0.667	29.5	33.9
2.00	.500	40.4	24.8
2.50	.400	51.2	19.5
3.00	.333	65.3	15.3
3.50	.286	83.3	12.0
4.00	.250	104.7	9.56
4.50	.222	136.0	7.35

[a] One milligram of ascorbic acid when oxidized is equivalent to 5.68×10^{-6} mole of quinone produced. [b] All chronometric end-points recorded in this communication are the average of three identical experiments. In all cases, the average deviation of a single observation from the mean (a. d.) was found to be not more than about ±1%.

The Correction Constant, M.—If, now, $1/Q$ (see Table I) is plotted as a function of $1/t$, the graphical method of Miller, *et al.*,[8] may be used to determine the values of the constants, a and b. In this method, the most probable straight line is drawn through the experimental points (see Fig. 1, solid line). From the slope and intercept of this line, (see Equation 1), it can be determined that in this experiment:

a = 16.55 mg. ascorbic acid
b = 297 seconds

If these values are substituted into Equation 2, it is possible to calculate the theoretical value of t for each value of Q (Q being known from the measured amount of ascorbic acid present in the system originally). Such calculated values of t are listed in the third column of Table II. Column 4 of this table shows the per cent. deviation between the calculated and observed values of t.

TABLE II

CALCULATED END-POINTS AND OBSERVED END-POINTS, WITH AND WITHOUT USE OF THE CORRECTION FACTOR, M

Q (mg. ascorbic acid)	t (sec., observed)	t (seconds, calculated) Neglecting M: t (sec.)	% deviation	Using M = 6.0 sec.: t (sec.)	% deviation
1.00	19.7	19.1	− 3.0	20.3	+3.0
1.50	29.5	29.8	+ 1.0	29.0	−1.7
2.00	40.4	40.8	+ 1.0	39.3	−2.7
2.50	51.2	52.9	+ 3.3	51.3	+0.2
3.00	65.3	65.9	+ 0.9	65.7	+0.6
3.50	83.3	79.5	− 4.6	82.9	−0.5
4.00	104.7	94.5	− 9.6	105.4	+0.7
4.50	136.0	110.9	−18.5	133.5	−1.8
Mean % deviation (ignoring signs)			5.2		1.4

It will be noted that the agreement between the calculated and observed values of t is poor for the

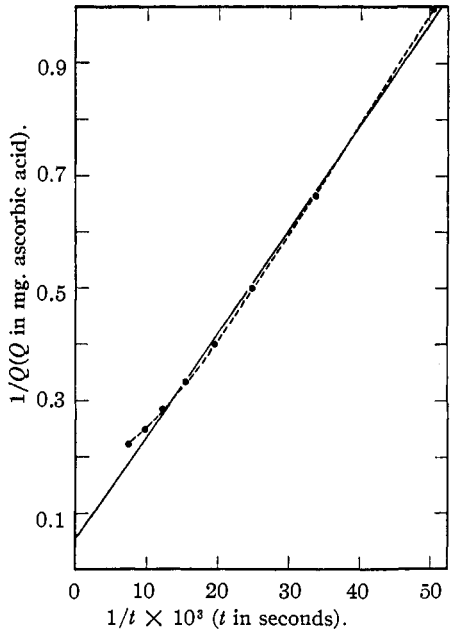

Fig. 1.—Showing the non-linearity of the relationship between the quinone formed, (Q), and the observed time of chronometric end-point, (t), when plotted reciprocally (*i. e.*, $1/Q$ *vs.* $1/t$). The plotted points are taken from the experimental data of Table I and the non-linearity of these end-points is indicated by the dotted line drawn through them. The solid line represents the most probable straight line through the observed points, using the method of least squares.

larger values of t. During the course of many such chronometric experiments, it became apparent that a very similar pattern of deviation made necessary a re-evaluation of the linearity of the $1/Q$–$1/t$ relationship (upon the experimental demonstration of which, it will be remembered, the chronometric equation was based).

The deviation "drift," referred to above, manifests itself in the $1/Q$–$1/t$ graph as a departure from linearity in the form of a shallow curve, convex toward the $1/t$ axis (see dotted line of Fig. 1).[18]

On examination by trial and error, however, it was found that if each t value was corrected by subtracting a constant (hereinafter symbolized as M), a linear relationship, within the limits of experimental error could be obtained.

In view of these results, Equation 2 may be modified in order to take into account the necessary correction of the observed end-point thus

$$Q = \frac{a(t - M)}{b + t - M} = \frac{aT}{b + T} \qquad (3)$$

The symbol T thus represents the "chronometric end-point" as distinct from the observed end-point, t. It is Equation 3 which will henceforward be referred to as the chronometric equation.

In order to solve Equation 3 for a, b and M, no simple graphical solution, comparable to that of Miller, *et al.*, is apparent. Instead, use must be made of more complicated algebraic solutions. Thus, if three observed Q–t relationships (Q_x, t_x; Q_y, t_y; Q_z, t_z) are chosen and substituted, each in its turn, into Equation 3, three relationships result, which may be solved simultaneously to yield a value for a

$$a = \frac{Q_z(t_z - t_y)(Q_y - Q_x) - Q_x(t_y - t_x)(Q_z - Q_y)}{(t_z - t_y)(Q_y - Q_x) - (t_y - t_x)(Q_z - Q_y)} \qquad (4)$$

With a known, two end-points can be used to solve for b, and with b known also, a single end-point suffices for M

$$b = (t_y - t_x)(a - Q_y)(a - Q_x)/a(Q_y - Q_x) \qquad (5)$$

$$M = (at - Q(b + t))/(a - Q) \qquad (6)$$

Applying Equations 4, 5 and 6 to the observed values of Q and t given in Table I, the following average values of a, b and M were obtained[19]

a	= 8.06 mg. ascorbic acid	(a. d. = ±6.4%)
b	= 100.7 seconds	(a. d. = ±2.6%)
M	= 6.0 seconds	(a. d. = ±8.2%)

Calculated values of t for each value of Q obtained by substituting the above values of a, b and M into Equation 3 are listed in Column 5 of Table II and the per cent. deviation of these from the observed values of t are listed in Column 6.

It is at once apparent that the agreement between the calculated and observed values of t using Eq. 3 is much better than that using Eq. 2 and approximates the experimental error. Similar results were obtained when a correction factor, M, was applied to the original data of all chronometric experiments made in this investigation.

The necessity for the use of this constant, M, in the chronometric equation would suggest that a measurable time was required for the initiation of the enzymatic reaction. That at least part of this short induction period is attributable to the finite time required for the complete mixing of the various components of the reaction mixture is indicated by the following.

In Table III are listed values of a, b and M, obtained in a manner similar to that described above, for enzyme reactions conducted at five different levels of aeration pressure, ranging from 0 to 7.62 cm.,[20] including the 2.54-cm. case already discussed in detail.

(18) On checking the previous data obtained in these laboratories by earlier workers, evidence was found of similar "drifts." These were not so marked, however, as those obtained in the present experiments, probably because no quantitative efforts had been made to control the extent of agitation due to the aeration stream, or to conduct experiments in the absence of agitation, where the "drift," as will be shown, is most apparent.

(19) In making these calculations, all eight points observed were substituted into Equation 6; all possible two-point combinations (*i. e.*, 28) were substituted into Eq. 5, and the averages taken. Because of the comparative complexity of Eq. 4, however, values of a reported here are averages of only 26 three-point combinations of the possible 56, the combinations being so chosen as to give as equal weight as possible to all observed endpoints.

(20) The equivalent of an aeration pressure of zero cm. (*i. e.*, absence of agitation) was obtained by shortening the air inlet tube so that it did not extend beneath the surface of the reaction mixture. In this way, air pressure was still available for starting the siphon.

TABLE III

VALUES OF a, b AND M AT VARYING AERATION PRESSURES

Aeration pressure, cm.	a, mg. of ascorbic acid	b, seconds	M, seconds
0	8.07 ± 0.45 (a. d.)	100.3 ± 3.2 (a. d.)	7.9 ± 0.8 (a. d.)
1.27	7.62 ± 0.55	92.4 ± 1.8	7.1 ± 0.9
2.54	7.95 ± 0.65	100.7 ± 2.7	6.0 ± 0.5
3.81	7.87 ± 0.44	99.7 ± 2.3	5.9 ± 0.7
7.62	8.06 ± 0.31	98.8 ± 1.6	5.2 ± 0.3
Mean value	7.95 + 0.19	97.4 + 1.0	

It can be seen that a and b show no significant trend in either direction as the aeration pressure is varied. These two constants may, therefore, be considered as independent of the degree of agitation within the limits tested. The value of M, however, is seen to decrease steadily as the degree of agitation is increased (assuming that the degree of agitation is directly proportional to the aeration pressure) so that the departure from the results predicted by Eq. 2 reaches a maximum in the absence of agitation. This state of affairs is consistent with the view that the induction period is due, at least in part, to a mixing effect.

Kinetic Implications of the Chronometric Equation.—Early in the course of the present investigation, it became apparent that an analysis of the chronometric equation in terms of the active enzyme present in the system at any given time showed promise of yielding worthwhile information concerning both the kinetics and the mechanism of the reaction inactivation phenomenon.

Thus, if it is assumed that the "active" enzyme, E, present in the reaction system at any "chronometric" time, T, is directly proportional to the rate of quinone formation at that time, then, from Eq. 3

$$E = k\frac{dQ}{dT} = \frac{kab}{(b + T)^2} \qquad (7)$$

The total enzyme, E_0, originally present in the reaction system is, similarly, proportional to the reaction rate at zero time

$$E_0 = k\left(\frac{dQ}{dT}\right)_{T=0} = \frac{ka}{b} \qquad (8)$$

Combining Eqs. 7 and 8

$$E = b^2E_0/(b + T)^2 \qquad (9)$$

Since the rate of enzyme inactivation is equivalent to the rate of disappearance of active enzyme with time, it follows from Eq. 9

$$-dE/dT = 2b^2E_0/(b + T)^3 \qquad (10)$$

From Eqs. 9 and 10, it is now possible to show that the chronometric equation implies an enzyme inactivation rate of 3/2 order with respect to enzyme, since

$$\frac{-dE/dT}{E^{3/2}} = \frac{2}{b\sqrt{E_0}} = \text{const.} \qquad (11)$$

It must be emphasized, however, that this conclusion is based on the assumption that the chronometric equation validly represents the actual course of enzyme inactivation with time. That it does so, within the limits of observational error, over the time interval which can be studied chronometrically, has been shown. However, the chronometric method is only applicable to about the first 140 seconds of the reaction[21] and, previous to this investigation, no information was available as to the course of the enzyme inactivation beyond that time. Actually, information regarding the later stages of the inactivation is critical to an evaluation of the kinetic order. If only the early stages of the inactivation are taken into consideration, a Q–T relationship implying a first-order mechanism fits the observed data as well as does the 3/2 order mechanism given above (Equation 3). Such a first-order relationship may be represented as

$$Q = a'(1 - e^{-T/b'}) \qquad (12)$$

This equation, referred to hereinafter as the "first-order equation," when analyzed by the methods used in Equations 8–11, yields the expression

$$(-dE/dT)/E = 1/b' = \text{constant} \qquad (13)$$

thus fulfilling first-order requirements.

The first-order equation may now be applied to typical chronometric data, and appropriate values obtained for the constants, a' and b' (the units of which are equivalent, respectively, to a and b in the chronometric equation). Thus it is found that if a' and b' are arbitrarily set equal to 5.24 and 69.7, respectively, and the equation is solved for T, using the experimental values of Q listed in Table I, the values thus obtained agree within experimental error with the observed values listed in Table I.[22]

Figure 2 indicates graphically the agreement between the observed results listed in Table I and the best available curves obtained from the chronometric equation and from the first-order equation.

It can be seen from Fig. 2 that over the time range available to the chronometric studies, no final decision can be made as to the relative validity of these two types of equations. In order to arrive at such a decision, an experimental technique was adopted which, while less precise than the chronometric technique, allowed enzyme activity measurements to be carried out for as long as twenty minutes after the initiation of the reaction.

The Aliquot Titration Method.—This technique involved an enzyme–catechol reaction conducted in the presence of a known excess of ascorbic acid. The course of the reaction was then followed by withdrawing a 10-ml. aliquot

(21) After intervals longer than about 140 seconds of reaction, the starch end-point becomes too faint for observation as a result of the decreasing concentration of enzyme, and hence of the decreasing rate of quinone formation.

(22) A satisfactory agreement is obtained in this manner only where the correction factor, M, (set equal, in this case, to 6.0 seconds, as determined above) is used to correct the observed end-points.

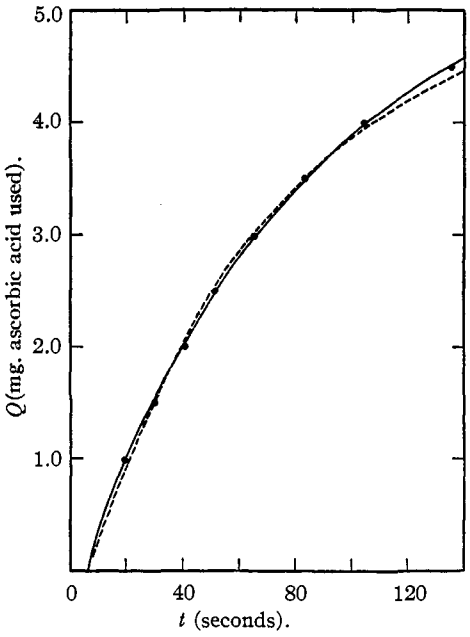

Fig. 2.—Showing the relationship between the quinone formed, Q, and the end-point, t, during the chronometric range (20–140 seconds). The plotted points are taken from the experimental data of Table I. The solid curve is calculated, using the chronometric equation (equation 3) and employing the average values of a, b and M described in connection with the discussion of equations 4, 5 and 6. The dotted curve is calculated, using the first-order equation (equation 12) and values of 5.24, 69.7 and 6.0 for a', b' and M, respectively (the values of a' and b' were selected by trial and error).

from the reaction mixture at a given time, stopping the enzyme reaction virtually instantly by transferring the aliquot immediately into 5 ml. of 2 N sulfuric acid, and then determining the quantity of ascorbic acid left unoxidized by titration with potassium iodate.[23]

The enzyme reaction was conducted in all respects according to the chronometric technique, except that ascorbic acid was used in excess, and that in the place of the siphon, an automatic levelling pipet was inserted into the third neck of the reaction flask. The pipet was used to extract an aliquot of constant volume which was then rapidly and reproducibly transferred into sulfuric acid by means of a wide-bore stopcock.

An air-inlet tube was used which did not extend below the surface of the reaction solution since, as shown in Table IV, the absence of agitation is an important factor in minimizing surface denaturation effects where comparatively long reaction times are under consideration.

In Table IV, the observed values of Q at reaction times of three minutes and of fifteen minutes are listed for values of aeration pressure varying from 1.27 to 3.81 cm. These are compared with the corresponding values obtained in the absence of agitation and with the theoretical values as calculated from the chronometric equation.

(23) R. Ballentine, *Ind. Eng. Chem., Anal. Ed.*, **13**, 89 (1941).

TABLE IV

EFFECT OF AERATION PRESSURE (DEGREE OF AGITATION) UPON THE VALUE OF Q AS OBTAINED BY THE ALIQUOT TITRATION METHOD

Aeration pressure, cm.	Q (mg. ascorbic acid oxidase) t equal to 3 minutes Value	% Deviation from theoretical	t equal to 15 minutes Value	% Deviation from theoretical
	5.14[a]		7.19[a]	
3.81	5.18	+0.8	5.90	−18.0
2.54	5.20	+1.2	6.10	−15.2
1.27	5.35	+4.1	6.35	−11.7
0	5.18	+0.8	6.71	− 6.7

[a] These are theoretical values for three minutes and for fifteen minutes as calculated from the chronometric equation (Eq. 3), using values of a and b shown in Table III. Values of a and b are independent of aeration pressure, and variations in M resulting therefrom are small when compared to relatively long reaction times of over three minutes.

It can be seen that the effect on Q of changing the degree of agitation in the reaction system is relatively small at t equal to three minutes. The variation in Q at this reaction time is within the estimated experimental error of the aliquot-titration method (=5%). At a reaction time of fifteen minutes, however, the cumulative effect of increased agitation is seen to be more important. Thus, with 3.81 cm. of aeration pressure, the discrepancy between the observed Q and the theoretical Q is 18%. With less agitation, the magnitude of this discrepancy decreases, until, in the absence of agitation, the discrepancy is only 6.7%.

The presence of ascorbic acid in excess showed no significant effect upon the tyrosinase–catechol reaction. Thus, where the ascorbic acid originally present was varied from 8 to 20 mg., the amount of ascorbic acid indirectly oxidized in a given time by a given quantity of enzyme and catechol was found to be constant within experimental error.

The data obtained during a typical aliquot titration experiment in which the enzymatic formation of quinone from catechol was followed for a period of twenty minutes (in the absence of agitation) are plotted as the experimental points in Fig. 3. The curves of Fig. 3 are an extension of the calculated curves obtained from the chronometric and first-order equations (see Fig. 2) and are included for comparison purposes.

Such a comparison argues strongly against the acceptance of the first-order equation as a valid representation of the Q–T relationship over the entire reaction course. It will be noted that the observed value of Q after twenty minutes of enzyme reaction is about 30% greater than that predicted by the first-order equation, a discrepancy far larger than can be accounted for by experimental error.

The disagreement of the observed values of Q with those predicted by the chronometric equation are much less serious. Whereas the discrep-

ancy between the observed Q and the first-order Q exceeds the experimental error within three minutes, the agreement of the observed Q and the chronometric Q falls within the experimental error for periods up to about eight minutes. It should be noted that at this reaction time (eight minutes) enzyme inactivation is 95% complete (as calculated from Equation 9, using a value of b equal to 100 seconds (see Table III)).

The relatively small discrepancy which is noted after eight minutes is not entirely unexpected. Dilute buffered solutions of tyrosinase, such as are used in enzyme activity experiments are known to inactivate on standing at about room temperature (25°) in the absence of substrate.[14,24]

Although such "environmental" inactivation may be minimized in the presence of substrate, it seems reasonable that a 6–8% discrepancy in the observed and calculated values of Q over a period of twenty minutes can be accounted for in terms of environmental inactivation superimposed on reaction inactivation.[25]

It may be concluded, therefore, that the chronometric equation represents the best expression for for the Q–t relationship available on the basis of present experimental knowledge.

Discussion

It has been shown above that the chronometric equation satisfactorily expresses the relationship between Q (quinone formed) and t (time of reaction) during virtually the entire course of the enzymatic oxidation of catechol. It therefore follows (see Equations 7–11) that the rate of enzyme inactivation at any time, t, is 3/2 order with respect to the active enzyme concentration at that time. This state of affairs means that the specific inactivation rate decreases with time in accordance with

$$\text{specific inactivation rate} = (-dE/dT)/E = 2/(b + t) \quad (14)$$

a relationship derived from Equations 9 and 10.

The dependence of the specific inactivation rate on the time of reaction suggests a "slowdown" departure from a first-order mechanism of enzyme inactivation. Several ways of accounting for such a "slowdown" process suggest themselves.

For instance, it may be considered that the inactive enzyme being formed, perhaps through a normally first-order mechanism, acts as a "protective protein" for the remaining active enzyme, thereby slowing down the subsequent inactivation process. Inert protein, such as gelatin,[24] is known to exhibit such a protective effect when present in relatively large concentration. However, the concentration of inert protein required for protection is of the order of 0.6 mg./ml., whereas the concentration of inactive enzyme in the chronometric reaction mixture is never higher than 0.15 microgram/ml. In view of this large difference in protein concentrations (1:4,000) it seems very doubtful that inactive enzyme can play a role as "protective protein."

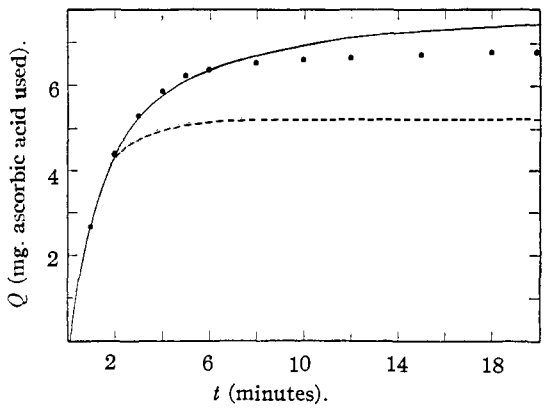

Fig. 3.—Showing the relationship between the quinone formed, Q, and the time of reaction, t, during the enzymatic oxidation of catechol as followed by the aliquot titration method. The plotted points are experimental. The solid and dotted curves are calculated from the chronometric and first-order equations, respectively, as in Fig. 2 except that the values of t have been extended to 20 minutes.

The possibility that various products of the catechol oxidation, *i. e.*, *o*-benzoquinone, its semiquinone intermediate, or quinone decomposition and polymerization products, may affect the course of the enzyme inactivation, has often been considered. However, in the chronometric method, *i. e.*, where ascorbic acid is always present, the decomposition and polymerization products of *o*-benzoquinone are never formed. The concentrations of *o*-benzoquinone itself (or its semi-quinone intermediate) that might conceivably exist momentarily on the enzyme surface are too small to be detectable by the very sensitive starch–iodide indicator. In view of the experiments of Ludwig and Nelson[6] with *o*-benzoquinone, it seems very unlikely that such small concentrations of the quinone can have a significant effect on the reaction course.

The possibility of a "slow-down" effect due to ascorbic acid itself, or to its degradation products, would also appear to be non-existent, since the course of oxygen uptake (as measured manometrically) has been shown, in these laboratories, to be unaffected by the presence or absence of ascorbic acid.

Finally, it should be noted that the possibility that short-lived intermediates formed in the "oxygen–water" phase of the total enzymatic oxidation–reduction cycle affect the reaction in any sig-

(24) W. H. Miller and C. R. Dawson, THIS JOURNAL, **63**, 3368 (1941).

(25) It will be recalled that the chronometric equation takes only reaction inactivation into account since its derivation is based entirely on experimental observations made during the very early phase of the reaction. It has been demonstrated that during this early phase of the reaction, environmental inactivation effects, such as that due to surface denaturation, are not significant (see Tables III and IV).

nificant way has been effectively eliminated by Roth.[26]

If consideration is now given to the possibility that the enzyme activity may be lost in stages rather than in a single-step process, another, and not heretofore considered, way of accounting for a "slow-down" departure from first-order kinetics becomes apparent. It is only necessary to propose a series of enzyme "intermediates" of successively lower activity and greater stability. The possible nature of such intermediates may now be considered.

It has been shown by Kubowitz,[27] and confirmed in these laboratories that tyrosinase activity is lost when the copper is removed from the protein. From the tyrosinase molecular weight data reported by Mallette and Dawson,[28] one may conclude that as many as four atoms of copper may be present per tyrosinase molecule. It is, therefore, quite conceivable that the reaction inactivation of the enzyme molecule might take place through the stepwise loss of copper atoms, with a corresponding loss of activity. Whether such partially-inactivated enzyme molecules would become more resistant to further inactivation, as would be necessary if a 3/2 kinetic order is to be accounted for, is, however, not known at present.

The nature of partially-inactivated tyrosinase "intermediates" may also be discussed from a slightly different angle. Tyrosinase has long been considered in these laboratories to be a single enzyme possessing two functions: that of catalyzing the oxidation of monophenols (*i. e.*, cresolase function) and that of catalyzing the oxidation of *o*-dihydric phenols (*i. e.*, catecholase function).[28] The relative rates at which a particular enzyme preparation catalyzes these two types of oxidation can be varied within certain limits, depending upon the method of isolation and purification of the enzyme.[9] It has been well-established that many properties of the enzyme are dependent upon the value of the catecholase/cresolase activity ratio of the preparation being tested.[28,29,30]

If it be postulated that the enzyme inactivation that occurs during the oxidation of catechol is the result of a step-wise preferential destruction of specific groupings on the enzyme molecule which are particularly concerned with the catecholase activity, the catecholase/cresolase activity ratio of the enzyme will decrease as the inactivation process continues. In this connection, the data presented by Roth, *et al.*,[31] are of particular interest for they show that tyrosinase preparations possessing a low catecholase/cresolase activity ratio are more resistant toward reaction inactivation than are preparations of high catecholase/cresolase activity ratios. From such considerations, it would follow that a stepwise destruction of catecholase activity centers might result in an increasing resistance of the enzyme system toward further inactivation, thereby leading to a deviation from first-order kinetics in the direction implied by the chronometric equation.

Whether such a stepwise mechanism, involving successive alterations in the enzyme molecule such as the loss of copper atoms or the loss of specific catecholase groupings, could account, *quantitatively*, for the observed experimental results may now be considered.

It will be noted that an inactivation model involving a series of successive steps, each first-order in nature, resembles kinetically the radioactive decay phenomena observed with certain radioactive families of elements. The kinetics of the decay of such radioactive families has been mathematically analyzed by Bateman[32] and such an analysis can be applied easily to the proposed enzyme inactivation model.

Thus, a simplified two-step version of this model may be pictured as

$$E_1 \xrightarrow{k_1} E_2 \xrightarrow{k_2} E_3$$

where E_1 is the original enzyme molecule, E_2 a partially inactivated form and E_3 a completely inactivated form, and where k_1 and k_2 are the respective first-order rate constants for the two steps of the inactivation.

If, now, the original concentration of E_1 is set equal to unity, and E_2 is R times as active as E_1, then E, the total enzyme activity at any time is

$$E = [E_1] + R[E_2] \qquad (15)$$

where $[E_1]$ and $[E_2]$ represent the concentrations of E_1 and E_2, respectively, at the given time. Using the Bateman equations to evaluate $[E_1]$ and $[E_2]$, Equation 15 becomes

$$E = e^{-k_1T} + \frac{k_1R}{k_1 - k_2}(e^{-k_2T} - e^{-k_1T}) \qquad (16)$$

Since, from chronometric relationships (see Equations 7 and 8)

$$E = b\mathrm{d}Q/a\mathrm{d}T \qquad (17)$$

where the enzyme concentration at zero time is unity, Equation 16 becomes

$$\mathrm{d}Q = \frac{a}{b}\left[e^{-k_1T} + \frac{k_1R}{k_1 - k_2}(e^{-k_2T} - e^{-k_1T})\right]\mathrm{d}T \qquad (18)$$

If Equation 18 is integrated from 0 to Q on the left and from 0 to T on the right, it is seen that

$$Q = \frac{a}{b}\left[\frac{1 - e^{-k_1T}}{k_1} + \frac{k_1R}{k_1 - k_2}\left(\frac{1 - e^{-k_2T}}{k_2} - \frac{1 - e^{-k_1T}}{k_1}\right)\right] \qquad (19)$$

Since, under the terms of the inactivation model here proposed, k_1 would represent the over-all spe-

(26) L. J. Roth, Dissertation, Columbia University, 1945.

(27) F. Kubowitz, *Biochem. Z.*, **292**, 221 (1937); **299**, 32 (1938).

(28) M. F. Mallette and C. R. Dawson, *Arch. Biochem.*, **23**, 29 (1949).

(29) M. H. Adams and J. M. Nelson, THIS JOURNAL, **60**, 2472 (1938).

(30) G. G. Parkinson and J. M. Nelson, *ibid.*, **62**, 1693 (1940).

(31) L. J. Roth, W. H. Miller and C. R. Dawson, *in press*.

(32) H. Bateman, *Proc. Cambridge Phil. Soc.*, **15**, 423 (1910).

cific inactivation rate (*i. e.*, $(-dE/dT)/E$) at zero time, when only E_1 would exist in solution, it follows from Equation 14 that k_1 can be set equal to $2/b$.

If, in accordance with the other requirements of the model (*i. e.*, that E_2 be less active and more stable than E_1), k_2 is made smaller than k_1 and arbitrarily set equal to $1/3b$ while the activity ratio, R, is arbitrarily set equal to 1/7, Eq. 19 can be evaluated to yield the relationship

$$Q = a(9.29 - 0.414e^{-2.000T/b} - 0.515e^{-0.33T/b}) \quad (20)$$

The excellent agreement between the Q–T curve calculated from this equation and that calculated from the chronometric equation is shown in Fig. 4. On the basis of the above reasoning, it would seem that the chronometric equation may be an empirical simplification of a relationship of the type

$$Q = a(C_0 - C_1e^{-K_1T/b} - C_2e^{-K_2T/b} - \ldots - C_ne^{-K_nT/b}) \quad (21)$$

where: (1) the number of exponential terms is equal to the number of first-order steps involved; (2) the values K_1, K_2, etc., are equal to bk_1, bk_2, etc. (where k_1, k_2k, etc., are the specific inactivation rates of the successive first-order steps involved); (3) the values C_0, C_1, C_2, etc., are each functions of the various K terms and of the relative activities of the various enzyme intermediates, such that $\sum_{i=1}^{i=n} C_i$ is equal to C_0; (4) the values of a and b are determined experimentally by means of the chronometric equation.

Acknowledgment.—It is a pleasure to acknowledge the coöperation of Mr. Stanley Lewis, who prepared the enzyme used in this investigation. The work was supported in part by a grant from the Division of Research Grants and Fellowships of the National Institutes of Health United States Public Health Service. The authors are indebted to Dr. Hans Lineweaver for suggesting an alternative interpretation of the data presented.

Summary

1. The aerobic oxidation of catechol, as catalyzed by the enzyme, tyrosinase, during the first 140 seconds of reaction has been carefully reinvestigated, using buffered enzyme–catechol systems containing limited quantities of ascorbic acid (the chronometric method). As a result, an earlier relationship used to express the total quinone formed as a function of time (*i. e.*, the "chronometric equation") has been modified by the introduction of a constant, M, which appears to represent, at least in part, a measure of the time of mixing of the various components of the reaction mixture.

2. By use of a modified technique of activity rate measurements in the presence of an excess of ascorbic acid and involving the titration of known aliquots of the reaction mixture at given times for ascorbic acid content, the course of the enzyme reaction was followed for periods up to twenty minutes. It was found that the reaction course as calculated from the chronometric equation agreed with the observed reaction course within experimental error for periods up to eight minutes, during which time enzyme inactivation was 95% complete.

3. An analysis of the kinetic implications of the chronometric equation has been presented and the results indicate that the specific inactivation rate of the enzyme is not constant but decreases with time in accordance with an inactivation process 3/2 order in respect to active enzyme. Several possible interpretations of these kinetic implications are inspected and arguments are presented against the possibility that this decrease in specific inactivation rate with time is due to the protective effect of compounds formed during the enzyme reaction.

4. It has been demonstrated that the course of the enzyme inactivation cannot be adequately represented by an equation derived on the assumption of a single-step, first-order inactivation process.

5. A kinetic model has been proposed in which the enzyme, tyrosinase, is considered as inactivating in stepwise fashion through a series of decreasingly stable intermediates.[33] It is argued

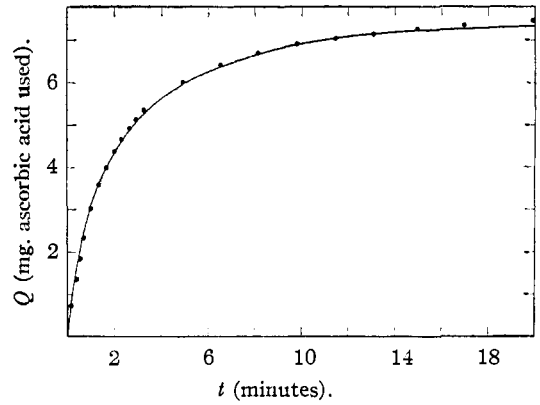

Fig. 4.—Showing the agreement between the chronometric equation and the two-step, first-order equation (equation 20) in predicting the Q–T relationship during the enzymatic oxidation of catechol. The solid line is calculated from equation 20 and the plotted points are calculated from the chronometric equation, using in each case the values of a, b and M given in Table III.

(33) In a personal communication, Dr. Hans Lineweaver has been so kind as to point out that "an equation of the same form as Equation 20 can be derived from the assumption that there are two enzymes (or one protein with two active groups) present that lose activity in a first-order manner at different rates. Whether the specific rates of action of the two "enzymes" are the same is not essential. These assumptions obviate the need for assuming that enzyme A is converted into B." Dr. Lineweaver's derivation follows: "Letting x and y stand for the two enzymes we have

$$-dx/dt = k_ax, \text{ and } -dy/dt = k_by$$

whence $x = x_0e^{-k_at}$, and $y = y_0e^{-k_bt}$. Now

$$dq/dt = k_1x + k_2y$$
$$= k_1x_0\,e^{-k_at} + k_2y_0\,e^{-k_bt}$$

or

$$q = \frac{k_1x_0}{k_a}(1 - e^{-k_at}) + \frac{k_2y_0}{k_b}(1 - e^{-k_bt})$$

or

$$= \left(\frac{k_1x_0}{k_a} + \frac{k_2y_0}{k^b}\right) - \frac{k_1x_0}{k_a}e^{-k_at} - \frac{k_2y_0}{k_b}e^{-k_at}$$

If Equation 17 is used, one obtains with the above assumptions

$$\frac{dq}{dt} = \frac{a}{b}(x_0e^{-k_at} + y_0e^{-k_bt})$$

or

$$q = \frac{a}{b}\frac{x_0}{k_a}(1 - e^{-k_at}) + \frac{a}{b}\frac{y_0}{k_b}(1 - e^{-k_bt})$$

Hence by comparison, the use of Equation 17 has made $a/b = k_1 = k_2$. Thus it would appear from this that the specific rates of action for the two active groups are the same. I believe it then follows by comparison with equation 20 that $x_0/y_0 = 4.8$ whereas $ka = 2.0/b$: and $kb = 1/3b$ as before." Although there is recent evidence[28] that the enzyme tyrosinase exists as a single protein, rather than a mixture of enzyme proteins, it is conceivable that two different catecholase, activity centers of different inactivation rates, might exist on the single protein at the start of the reaction. No experimental data are at hand, therefore, to allow for a choice between the interpretation of Dr. Lineweaver and that presented by the authors.

that such a model is not inconsistent with previously known facts concerning the inactivation properties of different types of tyrosinase preparations. A general equation derived from this kinetic model is presented, and it is suggested that the chronometric equation is merely an empirical simplification thereof.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, OREGON STATE COLLEGE]

Pantothenic Acid Studies. VII. N-Methylpantothenic Acid[1]

BY REX D. LINDSAY AND VERNON H. CHELDELIN

Pantothenic acid has been shown to exhibit a high degree of structural specificity for physiological action. The unnatural optical antipode, (−)pantothenic acid, is devoid of activity for bacteria[2] or rats,[3] and with the exception of "hydroxypantothenic acid,"[4] all structural analogs which have been prepared show dramatic reduction in activity. In addition, many of these analogs have been shown to competitively inhibit the action of the vitamin.

The comparative actions of various analogs of pantoic acid and pantothenic acid have been studied in microörganisms in an effort to locate possible points of attachment of the vitamin within the cell.[5] It has been suggested that this attachment normally takes place through the pantoic acid portion, since changes in this portion usually produce inert or slightly active analogs,[5] whereas alterations in the β-alanine moiety give uniformly good inhibitors for organisms requiring the preformed vitamin.[6-11] However, the function of the amide nitrogen has never been elucidated. The present study was undertaken to prepare N-methylpantothenic acid and to determine its growth promoting activity for different organisms.

The synthesis of N-methyl-β-alanine presumably could be accomplished readily by treating β-bromopropionic acid with methylamine.[12] However, this reaction invariably led to the simultaneous formation of amides, which in our experience could not be removed satisfactorily. Another possibility appeared through the addition of methylamine to acrylonitrile, but hydrolysis to the acid was unsuccessful. Alcoholic acid hydrolysis gave the (ethyl) ester of N-methyl-β-alanine, but in low yields. The desired compound was finally prepared as the ester in 35% yield through addition of methylamine to methyl acrylate.[13]

Syntheses of N-methylpantothenic acid (α,γ-dihydroxy-β,β-dimethylbutyryl-β′-N-methylalanide) were thus performed by condensing pantolactone with the methyl or ethyl ester of N-methyl-β-alanine, followed by saponification of the ester with barium hydroxide and removal of barium with sulfate. The product after purification was obtained as a viscous oil, which was best characterized as the brucine salt.

Experimental

Preparation of β-Cyanoethylmethylamine.—Acrylonitrile (21 g.) was slowly added with stirring and cooling to methylamine (15 g.) in methanol (65 g.).[14] Removal of the solvent and distillation gave the product (25 g.), b. p. 73° (16 mm.).

Ethyl β-Methylaminopropionate.—Hydrolysis of β-cyanoethylmethylamine (18 g.) with sulfuric acid (45 ml.) and ethyl alcohol (53 ml.) for five hours, gave 5 g. (18% yield) of the product, b. p. 66° (15 mm.).

(1) This material is taken from the thesis presented by R. D. L· for the degree of Master of Science, Oregon State College, 1949· The investigation was supported by research grants from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service and the Nutrition Foundation, Inc. Published with the Approval of the Monographs Publications Committee, Oregon State College, Research Paper No. 139, School of Science, Department of Chemistry. A preliminary report was presented before the Pacific Coast convention of Phi Lambda Upsilon, Corvallis, Oregon, February 18-19, 1949.

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(11) E. E. Snell and W. Shive, *ibid.*, **158**, 551 (1945).

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