The hypoiodite consumption of a one-gram sample of the hydrocelluloses under conditions analogous to the preparative method was equivalent to 4.47 ml. of 0.02 *N* thiosulfate or 0.045 mM/g. The consumption of methanolyzed cellulose under the same conditions was 0.003 mM/g., this figure being indicative of the amount of secondary oxidation to be expected. Increasing the time of oxidation from 4 to 6 hours caused no additional consumption of hypoiodite in excess of that consumed by the blank. From these figures the number-average degree of polymerization of the oxycellulose is calculated to be 140.

The oxidized cellulose contained 0.049 mM carboxyl per gram as measured by the back-titration method of Neale and Stringfellow[10] and 0.045 mM/g. by methylene blue absorption.[11] The methylene blue absorption figure for the hydrocellulose prior to oxidation was 0.002 mM/g.

Isolation of D-Gluconic Acid.—Methanolysis of 125 g. of oxycellulose in methanolic hydrogen chloride (initially, 0.5 *N*; finally, less than 0.01 *N*) for 2 hr. at 120° solubilized about 3% of the material. The insoluble residue was washed with ethanol until acid-free and with ether. Weight of residue, 121 g.; carboxyl by back-titration 0.028 mM/g., carboxyl by methylene blue consumption, 0.029 mM/g.; ester methoxyl, 0.003 mM/g. calculated from a colorimetric determination of methanol[12] after alkaline saponification.

The soluble portion of the methanolyzate with washings was neutralized with ethereal diazomethane and was concentrated under reduced pressure. The concentrate yielded 1.87 g. of crystalline methyl glucosides in three crops from absolute alcohol. The mother liquor, containing 1.30 g. of solids, was diluted to 25 ml. and aliquots were used for further investigations.

A 5-ml. aliquot was concentrated in a stream of air to about 1/2 ml. and to this solution was added 100 mg. of phenylhydrazine in 1/2 ml. of absolute ethanol. After a few hours at 5° D-gluconic phenylhydrazide began to precipitate, the yield after 2 days being 0.066 g., m.p. 200–202°.[13] One recrystallization from hot water increased the melting point to 204–205°, which value was not changed by admixture with the phenylhydrazide prepared from D-gluconic γ-lactone. The specific rotation of the isolated material was $[\alpha]^{25}D$ +12° (*c* 1, water, 1-dm.), identical with the literature value.[14] On the basis of this yield, the entire methanolysate would contain 0.33 g. of phenylhydrazide, equivalent to 0.23 g. of D-gluconic acid; the recovery amounts to 60% based on the difference in carboxyl analyses before and after methanolysis.

Aliquots treated with alcoholic ammonia and with alcoholic methylamine, respectively, produced in lower yields crystalline materials with melting points not changed by admixture with authentic specimens of the respective amides but depressed by either the α- or β-form of methyl D-glucopyranoside. Second crops were contaminated by glucosides.

Preparation of D-Gluconic Methylamide.—A solution of 100 mg. of D-gluconic γ-lactone in 1 ml. of methanol saturated with methylamine deposited during 24 hr. at 5° the crystalline amide; weight, 0.111 g. (95% of the theoretical yield), m.p. 150.5–151.5°. Constants determined after one recrystallization from 95% ethanol were m.p. 152–153°, $[\alpha]^{26}D$ +39.9° (*c* 4, water, 2-dm.) unchanged by a second recrystallization. van Wijk[7] reports m.p. 127°; $[\alpha]^{25}D$ +33.8°; N, 6.49.

Anal. Calcd. for $C_7H_{15}O_6$: C, 40.19; H, 7.22; N, 6.69. Found: C, 40.13; H, 6.92; N, 6.65.

(10) S. M. Neale and W. A. Stringfellow, *Trans. Faraday Soc.*, **33**, 881 (1937).

(11) G. F. Davidson, *Shirley Institute Memoirs*, **21**, 47 (1947).

(12) C. L. Hoffpauir and R. E. Reeves, *Anal. Chem.*, **21**, 815 (1949).

(13) All melting points were determined with a Fisher-Johns micro-melting-point apparatus.

(14) K. Rehorst, *Ber.*, **61B**, 163 (1928).

NEW ORLEANS, LOUISIANA

[CONTRIBUTION FROM THE FRUIT PROCESSING DIVISION, WESTERN REGIONAL RESEARCH LABORATORY]

Enzymatic Browning of Fruits. III. Kinetics of the Reaction Inactivation of Polyphenoloxidase

BY LLOYD L. INGRAHAM, JOSEPH CORSE AND BENJAMIN MAKOWER

RECEIVED NOVEMBER 30, 1951

The inactivation of polyphenoloxidase which accompanies the aerobic oxidation of the substrate, catechol, has been studied at various enzyme concentrations by the usual method of following the concentration of a secondary reductant, ascorbic acid. The amount of ascorbic acid oxidized at any chosen reaction time and the maximum amount of ascorbic acid oxidized are both proportional to the initial enzyme concentration when the latter is varied over a 5-fold range. Also, the time for half the maximum amount of ascorbic acid to be oxidized is independent of the initial enzyme concentration. These data are consistent with first order kinetics with respect to enzyme concentration rather than a three-halves order kinetics previously proposed.

Introduction

Dawson and co-workers[1] developed a valuable method for measuring the activity of polyphenoloxidase. It is based on the secondary oxidation of ascorbic acid by the quinone formed in the enzymatic oxidation of the catechol. Since the enzyme is inactivated during the course of the reaction, the rate of the reaction decreases with time. This inactivation has been termed "reaction inactivation."[2] These authors found that the data could be represented by the "chronometric equation"

$$Q = at/(b + t) \qquad (1)$$

where Q is the amount of quinone produced, t is the time and a and b are experimentally determined constants. This equation represented the data so well over 80% of the reaction that Asimov and Dawson[3] sought to find its theoretical significance. They showed that the equation could be derived by assuming that the inactivation was proportional to the three-halves power of the enzyme concentration. They also considered the possibility of a first order enzyme dependence which leads to the equation

$$Q = a'(1 - e^{-t/b'}) \qquad (2)$$

This equation was rejected on the basis that Equation 1 fitted the experimental data (then available) better during the latter part of the reaction than Equation 2, when values of the parameters, $a' = 8.06$ mg. ascorbic acid and $b' = 100.7$ sec. were chosen. These values were determined over the more precise portion of the Q–t curve, that portion under two minutes.

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

(2) W. H. Miller and C. R. Dawson, *ibid.*, **68**, 3378 (1941).

(3) I. Asimov and C. R. Dawson, *ibid.*, **72**, 820 (1950).

However, by choosing different parameters of $a' = 6.60$ mg. of ascorbic acid and $b' = 113$ sec., it is shown in Figs. 1 and 2 that Equation 2 appears to fit the whole range of experimental data as well as Equation 1. It is apparent, therefore, that this method of judging between the two kinetics is not conclusive.

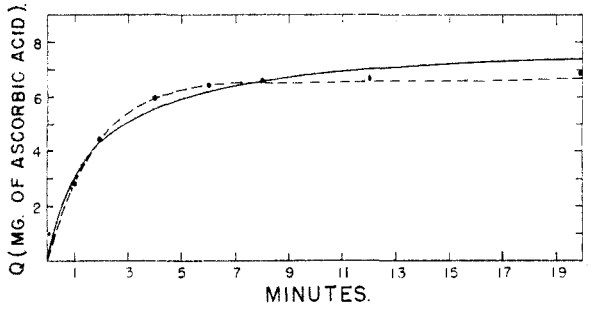


Fig. 1.—The Q dependence upon t. The circles are the experimental points given by Asimov and Dawson and the solid line is their best fit of Equation 1 to these data. The dashed line represents Equation 2 using values of $a' = 6.60$ mg. of ascorbic acid and $b' = 113$ sec.

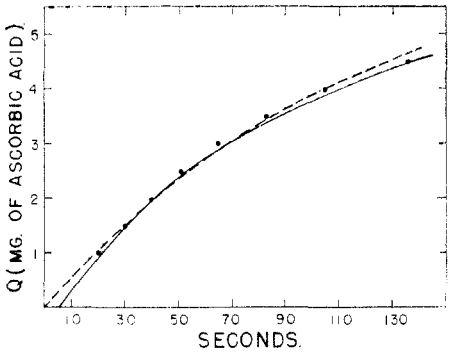


Fig. 2.—The Q dependence upon t during the chronometric region (see legend to Fig. 1).

To arrive at a more confident value for the order of the enzyme in the inactivation reaction, it was decided to analyze the oxidation kinetics by other means. Instead of measuring Q as a function of time at a single value of initial enzyme concentration, E_0, a study was made in several reaction mixtures differing only in E_0. In this manner it was possible to determine the effect of varying E_0 on (1) the maximum amount of ascorbic acid oxidized during the reaction, on (2), the half-life of the enzyme, and on (3), the pseudo half-life of the ascorbic acid (the time for half of the maximum amount of ascorbic acid to be oxidized). The relation between E_0 and the three quantities mentioned above was then analyzed in terms of the first order and the three-halves order kinetics. From this comparison it was concluded that the experimental evidence is greatly in favor of the first order reaction. It is perhaps pertinent at this point to note that if the equation for the first order dependence (equation 2) is expanded into a series and if all of the higher terms of the series are dropped, the equation

$$Q = a't/(b' + t)$$

is obtained which is the same as equation 1.[4] Hence, we see why the chronometric equation is really a good approximation of the course of the first portion of the reaction.

Experimental

The experimental details for both the chronometric and aliquot titration methods are essentially the same as those described by Asimov and Dawson[3] except for differences in aeration and addition of enzyme as noted below. In the aliquot titration method, instead of using an unagitated reaction mixture, the solution was aerated with 1 cm. air pressure. This was done to eliminate any possibility of insufficient supply of oxygen during the course of the reaction. Though some denaturation of the enzyme may result from the aeration, the resulting error was estimated from the data of Asimov and Dawson[3] to be not greater than 5%. The enzyme was added last to minimize the denaturation. Time was measured from the addition of the enzyme (0.4 to 2.0 ml. from a 2-ml. graduated pipet). Two polyphenolase preparations were used, one a commercial preparation from mushrooms[5] diluted with ice-water to a concentration of 22 catecholase units per ml., the other a sample of apple enzyme (prepared by Dr. J. D. Ponting of this Laboratory) with an activity of 19 catecholase units/ml. The optimum catechol concentration was found to be 30 mg. of catechol for the mushroom enzyme and 160 mg. per 100 ml. of solution for the apple enzyme. Water redistilled through an all-glass apparatus was used in all experiments. The experimental data are given in Tables I and II.

TABLE I

TIME IN MINUTES FOR OXIDATION OF A GIVEN AMOUNT OF ASCORBIC ACID IN CHRONOMETRIC EXPERIMENTS WITH MUSHROOM ENZYME

Ml. enzyme[a] → Mg. ascorbic acid	0.4	0.8	1.2	1.6	2.0
0.4	0.50				
0.7	0.90				
0.9		0.49	0.30	0.20	
1.1	1.33				
1.3	1.66				
1.4		0.70			
1.5	2.30				
1.8		1.00	0.63	0.42	0.28
2.7		1.40	1.00	0.72	
3.6		2.30	1.40	1.03	0.63
4.6			2.40	1.39	0.94
5.5				1.85	1.10
6.4					1.47
7.3					1.74

[a] Commercial mushroom enzyme diluted to 22 catecholase units/ml.

TABLE II

MG. OF ASCORBIC ACID USED IN ALIQUOT TITRATION EXPERIMENTS WITH MUSHROOM ENZYME

Time, min. → Ml. enzyme[b]	1[a]	2[a]	4	8	12	20
0.4	0.9	1.5	1.8	1.9	2.0	2.1
0.8	2.1	3.5	3.7	3.9	4.0	4.3
1.2	3.0	4.4	5.4	5.5	5.7	6.1
1.6	3.7	5.8	7.8	8.1	8.4	8.5
2.0	4.8	7.8	9.7	10.1	10.2	10.4

[a] The values of Q at one and two minutes were interpolated from the chronometric data. [b] Commercial mushroom enzyme diluted to 22 catecholase units/ml.

Results and Discussion

The usual method used to determine kinetics is

(4) We are indebted to Dr. Hans Lineweaver of this Laboratory for pointing this out to us.

(5) Obtained from the Worthington Biochemical Co., Freehold, N. J.

to study the effect that variations in the concentration of the reactant in question have on the initial reaction rate. However, in the case under consideration this is impossible because it is only practical to study Q and not the enzyme activity, E, as a function of time. The initial rate of appearance of Q is the same from both equations 1 and 2

$$(dQ/dt)_{t=0} = \frac{a}{b} = \frac{a'}{b'} = k_0E_0 \qquad (3)$$

In order to determine the effect of variations in E_0 upon the reaction, as predicted by the two kinetic theories, the constants in equations 1 and 2 have been evaluated, in terms of E_0 and various rate constants. This was done by integrating the three-halves order and the first order equations for the reaction inactivation $dE/dt = -k_IE^{3/2}$ and $dE/dt = -k'_IE$ to obtain $E = 4E_0/(2 + E_0^{1/2}k_It)^2$ and $E = E_0 \exp.(-k'_It)$, respectively. Using these values of E to integrate

$$dQ/dt = k_0E \qquad (4)$$

we obtain for the three-halves order case

$$Q = \frac{2(k_0/k_I)\,E_0^{1/2}t}{2(E_0^{-1/2}/k_I) + t} \qquad (1a)$$

and for the first order inactivation

$$Q = (k_0E_0/k'_I)(1 - e^{-k'_It}) \qquad (2a)$$

where k_0, k_I and k'_I refer to the specific rate constants for oxidation, three-halves order inactivation, and first order inactivation, respectively. These two equations will now be used to interpret the experimental results.

Effect of E_0 on the Maximum Amount of Ascorbic Acid Oxidized.—The maximum amount of ascorbic acid oxidized during the reaction is found to be

$$\lim Q_{t\to\infty} = 2k_0E_0^{1/2}/k_I \qquad (5)$$

if equation 1a is valid, and

$$\lim Q_{t\to\infty} = k_0E_0/k'_I \qquad (6)$$

if equation 2a is valid. Thus, three-halves order inactivation kinetics would predict $(Q/\sqrt{E_0})_{t\to\infty}$ to be constant with a variation in E_0 whereas first order kinetics would predict $(Q/E_0)_{t\to\infty}$ to remain constant. Since Q is not increasing with time at 20 min., the value of Q at 20 min. has been taken as $\lim Q_{t\to\infty}$. In columns 7 and 8 of Table III are shown the ratios between Q at 20 min. and E_0 and $\sqrt{E_0}$. The near constancy of Q/E_0 contrasted with the over 2-fold variation in $Q/\sqrt{E_0}$ when E_0 is varied 5-fold is in agreement with the first order kinetics. Similar conclusions can be drawn from the data shown in Table IV obtained with a sample of polyphenoloxidase from apples.

TABLE III

TEST OF FIRST ORDER KINETICS

Min. →	1	2	4	8	12	20	20	
Enzyme,[b] ml.	Q/E_0[c]	Q/E_0	Q/E_0	Q/E_0	Q/E_0	Q/E_0	$Q/\sqrt{E_0}$	P,[a] min.
0.4	2.2	3.7	4.5	4.8	5.0	5.3	3.3	1.25
0.8	2.6	4.4	4.6	4.9	5.0	5.4	4.8	1.15
1.2	2.5	3.7	4.5	4.6	4.8	5.1	5.6	1.10
1.6	2.3	3.6	4.9	5.1	5.2	5.3	6.6	1.25
2.0	2.4	3.9	4.9	5.1	5.1	5.2	7.4	1.10

[a] Pseudo half-life interpolated from the chronometric data. The maximum value of Q was taken as its value at 20 minutes. [b] Commercial mushroom enzyme diluted to 22 catecholase units per ml. [c] In units of mg. ascorbic acid oxidized per ml. of enzyme.

TABLE IV

VARIATION OF ASCORBIC ACID OXIDIZED AFTER 20 MINUTES WITH AMOUNT OF APPLE ENZYME

Enzyme, E_0,[a] ml.	Ascorbic acid[b] oxidized, mg.	Q/E_0	$Q/\sqrt{E_0}$
0.5	3.7	7.4	5.2
1.0	7.4	7.4	7.4
2.0	14.3	7.2	10.1

[a] Apple enzyme, 19 catecholase units/ml., prepared by Dr. J. D. Ponting. [b] A total of 25 mg. of ascorbic acid was used in all experiments.

Effect of E_0 on the Half-life of the Enzyme.—Another method to evaluate the kinetics is to investigate the effect that variations in E_0 have upon the half-life of the enzyme. The obvious method to study the half-life would involve a study of the slope of the Q–t curve, since the slope is a measure of the enzyme activity (*cf.* Equation 4). Because of the inherent difficulties in determining the slope of an experimental curve we have chosen a less complicated way to find the dependence of the half-life of the enzyme on the original enzyme concentration.

If it can be shown experimentally that Q is proportional to E_0 at any chosen time, t, as measured in several reaction mixtures differing only in E_0, then the half-life of the enzyme can be proven to be independent of E_0. If Q is proportional to E_0 then

$$Q = E_0f(t) \qquad (7)$$

where $f(t)$ is the dependence Q has upon t for unit enzyme activity. Differentiating with respect to time and combining with Equation 4 we obtain

$$k_0E = E_0(d[f(t)]/dt)$$

When $E = E_0/2$ and $t = t_{1/2}$ (half-life time)

$$d[f(t_{1/2})]/dt = k_0/2$$

showing that if Q is proportional to E_0 at any measured time then the enzyme half-life is independent of E_0 and the reaction inactivation is first order with respect to E.

In Table III, columns 2–7, are listed the mg. of ascorbic acid oxidized per ml. of enzyme used, Q/E_0, at six chosen times between 1 and 20 min. with a 5-fold variation in E_0. The near constancy of this ratio at any given time is shown to hold over the entire course of the reaction in agreement with the predictions of first order kinetics. If the kinetics were three-halves this would only be true when $t \ll b$ (hence for about the first 10 sec. of the reaction).

Effect of E_0 on the Pseudo Half-life of the Ascorbic Acid.—The effect of variations in E_0 upon the pseudo half-life serves as another test for differentiating between the two considered inactivation kinetics. The pseudo half-life is the time for half of the maximum amount of ascorbic acid to be oxidized. From Equation 1a the pseudo half-life, P, is found to be

$$P = 2/k_1\sqrt{E_0} \qquad (8)$$

whereas if Equation 2a is valid the pseudo half-life is

$$P = \ln 2/k_I' \qquad (9)$$

The pseudo half-lives have been taken as the times graphically interpolated from the chronometric data when Q is one-half the value of Q at 20 minutes. The values of P are shown in the last column of Table III, at various values of E_0, to be nearly constant for a 5-fold variation in E_0. This is in agreement with the predictions of Equation 9 for the first order kinetics.

Conclusions.—The experimental evidence is consistent with first order kinetics for enzyme inactivation and is not consistent with three-halves order kinetics. It is thus no longer necessary to assume the stepwise inactivation mechanism proposed by Asimov and Dawson but there is insufficient evidence at the present time to propose another mechanism. Regardless of the kinetic interpretations, the usefulness of their chronometric equation for assay purposes remains unimpaired.

Acknowledgment.—We wish to thank Dr. Hans Lineweaver and Dr. Sigmund Schwimmer for helpful discussions concerning this research.

ALBANY, CALIFORNIA

[CONTRIBUTION FROM THE BASIC SCIENCES RESEARCH DEPARTMENT, U. S. NAVAL CIVIL ENGINEERING RESEARCH AND EVALUATION LABORATORY]

The Reaction of Certain Substituted Furfurals with Aniline and Aniline Hydrochloride

BY RICHARD W. DRISKO AND HERBERT MCKENNIS, JR.

RECEIVED OCTOBER 15, 1951

An investigation has been conducted to determine whether a number of substituted furfurals in reaction with aniline and aniline hydrochloride undergo ring cleavage as does furfural. Both 5-chloro-2-furaldehyde and 5-bromo-2-furaldehyde yielded bis-(phenylamino) compounds, and no cleavage of the furan nucleus of the parent aldehydes was noted. 5-Nitro-2-furaldehyde, under the same conditions, gave the corresponding anil in good yield. Thiophene-2-aldehyde was found to combine with aniline to form an anil which did not react further with aniline hydrochloride.

Since Stenhouse[1] first reported that aniline, aniline hydrochloride and furfural react together to form a deep purple compound, the structure of this compound and the mechanism of its formation have had widespread investigation.[2-8] The evidence thus far indicates that the formula (I) of Zincke and Mülhausen[4] is consistent with the properties of the compound

$$(C_6H_5NH_2CH{=}CHCH{=}C(OH)CH{=}NC_6H_5)^+Cl^-$$
I

Youngburg and Pucher[9] found that methyl-2-furaldehyde[10] reacts with aniline acetate to give a light yellow color that does not appreciably interfere in the determination of furfural. Experiments in our laboratory have shown that aniline hydrochloride reacts slowly with 5-methyl-2-furaldehyde in ethanol to form a deep red color. The anil of 5-methyl-2-furaldehyde has been prepared and has also been found to react slowly with aniline hydrochloride to form a deep red color. This suggested that certain nuclear substituted furaldehydes and their anils would undergo ring cleavage in a manner similar to the cleavage of furfural itself.

When 5-chloro-2-furaldehyde and 5-bromo-2-furaldehyde were treated with aniline and aniline hydrochloride, an entirely different reaction was found to occur. It was further noted that the same product was formed when two moles of aniline was used instead of one mole each of aniline and aniline hydrochloride.

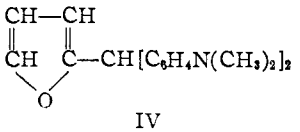
In considering the possible structures of the products obtained, it was noted that Schmid and Becker[11] reported that acetaldehyde and propionaldehyde react with 2-aminopyridine in alcohol to form products which gave the correct analyses for compounds which may be assumed to have formulas II and III, although a rigid proof of structure was not given.[12,13]

$CH_3CH(NHC_5H_4N)_2$ II

$CH_3CH_2CH(NHC_5H_4N)_2$ III

A similar reaction has been reported by Kondo and Ochiai[14] who found that methylal combines with β-phenylethylamine in the presence of hydrochloric acid to form diphenylethylaminomethane.

Fischer[15] has found that dimethylaniline reacts with furfural in the presence of zinc chloride to form a compound (IV) of the triphenylmethane type.



IV

The absorption spectra of the compounds obtained from the 5-halo-2-furaldehydes and aniline (Fig. 1) suggested the same type of structure for the two compounds. Elementary analyses showed

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(3) W. König, *J. prakt. Chem.*, **72**, 555 (1905).
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