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A New Method for the Measurement of Tyrosinase Catecholase Activity. II. Catecholase Activity Based on the Initial Reaction Velocity

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In a previous publication[2] a new technique for the determination of the catecholase activity of the phenol oxidizing enzyme, tyrosinase, was described. This technique was referred to as the chronometric method since it involves the measurement of the time necessary for a given quantity of the enzyme to produce *o*-benzoquinone in the catechol–enzyme system just in excess of that necessary to oxidize a small amount of ascorbic acid also present in the system. The end-point time, time of appearance of excess *o*-benzoquinone, is indicated by a blue coloration at the point where the reaction mixture is continuously sampled dropwise into an acidified starch–iodide indicator solution.

Because it seemed advisable for purposes of comparison to continue the activity data in a form previously used in this and other laboratories, the chronometric method was calibrated against the direct manometric method of measuring the catecholase activity. That is, the end-point times obtained using various amounts of the enzyme with a prescribed amount of ascorbic acid in the system were correlated with the activity of the enzyme as based on oxygen uptake measurements made during the enzymatic oxidation of catechol in the absence of ascorbic acid. A simple equation was thus given for the calculation of the activity from the end-point time, based on the calibration value of 34 catecholase units for an end-point of sixty seconds using a 250-cc. reaction volume. This value (34 units) was obtained after a statistical treatment of a large number of one minute reading experiments using an appropriate dilution of the enzyme in the direct manometric method.[3] It was pointed out that such activity measurements made by the chronometric method were much more reproducible than those obtained using the manometric method and could be obtained with considerably less effort.

A difficulty in obtaining a reliable measure of the catecholase activity of tyrosinase with the usual Warburg manometer technique rests on the fact that during the enzymatic oxidation of catechol the enzyme frequently is very rapidly inactivated. In such a case the course of the oxygen uptake is at no time linear in form, and rate measurements are therefore difficult to obtain. This difficulty generally becomes more and more evident as the purity of the enzyme, or freedom from extraneous inactive protein material, is increased. The limitations of the respirometer method are such that accurate readings of oxygen uptake are not possible during the initial phases of the reaction, hence rate measurements during the first two to three minutes of the reaction are subject to an experimental error estimated by Parkinson and Nelson[4] to be about 20–30%. On the other hand, end-point times by the chronometric method can be obtained with good precision at any time within the interval of twenty to one hundred and fifty seconds after initiating the enzymatic oxidation of catechol.

An application of the chronometric method to a study of this inactivation of the enzyme occurring during the initial phases of the enzymatic oxidation of catechol reveals that the different types of tyrosinase preparations[5] differ markedly in the degree of inactivation of the enzyme that occurs during the first sixty seconds of the reaction.[3] This of course means that catecholase activities based on rate measurements made after the reaction has progressed for a short time are influenced by the degree of enzyme inactivation that has occurred up to that time. In other words, a measurement of the rate of oxidation (activity) made at sixty seconds using a preparation that does not inactivate during this time will give a true value of the original activity of the enzyme. However, the measurement made at sixty seconds for a preparation that has undergone serious inactivation during the first minute of the reaction will, of course, yield an activity value lower than the true activity of the original enzyme preparation.

It is apparent that to compare on a common basis the catecholase activities of tyrosinase preparations differing in the degree of enzyme inactivation that occurs during the initial phases of the enzymatic oxidation of catechol, the activities must be based on the initial reaction velocity. In terms of oxygen uptake, or in terms of *o*-benzo-

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

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

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

(5) Preparations of tyrosinase from the common mushroom *Psalliota campestris* vary in their ratio of catecholase to cresolase activity depending on the method of isolation and purification employed. Those which are relatively much more active with catechol than with *p*-cresol have been termed high catecholase preparations to distinguish them from the so-called high cresolase preparations in which the two activities are more nearly comparable. These preparations have properties that are quite different[3,4,6,7] and the question of whether or not they are mixtures of two enzymes, or result because of modification of one enzyme protein during processing, etc., has not yet been definitely settled.

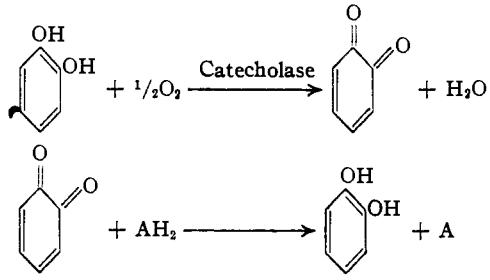
(6) W. H. Miller and C. R. Dawson, THIS JOURNAL, **63**, 3368 (1941).

(7) W. H. Miller and C. R. Dawson, *ibid.*, **64**, 2344 (1942).

(8) To be published.

quinone production, the initial reaction velocity is dO_2/dt or dQ/dt where $t \cong 0$. The lack of precision inherent in the manometric method makes the determination of dO_2/dt at $t \cong 0$ impracticable, but a slight modification of the chronometric technique as previously described yields the value of dQ/dt at $t \cong 0$ with good precision. It is the purpose of this communication, therefore, to describe how the true catecholase activity of the enzyme, based on the initial reaction velocity and hence not influenced by the inactivation characteristics of the preparation, may be obtained using the chronometric method.

The Initial Reaction Course.—As previously indicated, when catechol is enzymatically oxidized in the presence of ascorbic acid,[9] no detectable amount of *o*-benzoquinone exists in the solution, and the reaction mixture remains perfectly colorless until the moment the ascorbic acid (AH_2) has all been converted to dehydroascorbic acid (A)



If the experiment is set up a number of times using different amounts of ascorbic acid with a given amount of enzyme and catechol, a series of end-point times (time of appearance of *o*-benzoquinone in system) is obtained. In this way the production of *o*-benzoquinone (or its equivalent—the disappearance of ascorbic acid) can be measured as a function of time for a given amount of the enzyme. The method is, therefore, nothing more than an indirect titration of the *o*-benzoquinone, performed in such a manner that the quinone is never present in the reaction mixture until the instant of its detection. This eliminates from consideration the many complex side reactions which make the direct titration of *o*-benzoquinone unsuitable for measuring the enzyme activity.[11] By using small amounts of ascorbic acid in the system the *o*-benzoquinone production can be followed during the very early stages of the reaction with good precision.

The data given in the form of Curves I, II and III in Fig. 1 show, in terms of quinone production, the initial reaction course during the enzymatic oxidation of catechol as catalyzed by three

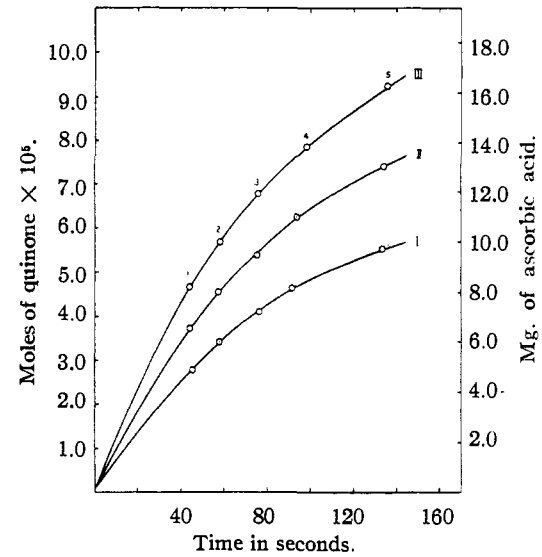


Fig. 1.—Curves showing the production of *o*-benzoquinone during the initial phase of the enzymatic oxidation of catechol. Aerobic oxidation catalyzed by three different amounts of a purified high catecholase preparation of tyrosinase.[12] Each experimental point obtained and checked (see experimental section) using a 250-cc. reaction volume containing 50 mg. of catechol (optimum) and ascorbic acid as shown; *p*H 5.5; temp., 25°. Enzyme employed was tyrosinase preparation C143F1, diluted 1:10 and then this sub-dilution further diluted 1:100 just before using; total dilution 1:1000. Curve I, 6 cc.; Curve II, 8 cc.; Curve III, 10 cc. The undiluted enzyme had a dry wt. of 5.9 mg./cc.,[13] contained 0.07% Cu,[14] 475 cresolase units/cc.,[3] and was stable for a long period when stored at refrigerator temperature. In the 1:10 dilution it was stable for several days at low temperature. See experimental section for method of obtaining data.

different amounts of a purified, typical, high catecholase preparation of tyrosinase.[5] Besides enzyme and ascorbic acid, as shown, the original reaction mixtures for each experimental point contained catechol in optimum concentration for this type enzyme preparation, *i. e.*, 50 mg. in a 250-cc. reaction volume, added at zero time to the buffered enzyme–ascorbic acid mixture (*p*H 5.5) to initiate the reaction. The oxidations were carried out at 25°. Other experimental details are given in the legend of the figure and the experimental section.

Because the catechol concentration remains constant during the reaction and is present in large excess in comparison to the amount of *o*-benzoquinone formed, the drop in rate of production of quinone that is evident in each case can only be attributed to serious inactivation of the enzyme in the early stages of the reaction.

(9) The ascorbic acid functions only as a reducing agent and does not significantly inhibit or accelerate the enzyme action. Its oxidation is not catalyzed by the enzyme under these conditions, nor does it affect the inactivation of the enzyme.[3,10]

(10) B. J. Ludwig and J. M. Nelson, THIS JOURNAL, **61**, 2601 (1939).

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

(12) Prepared from the common mushroom, *Psalliota campestris*, by a modification (to be published later) of the method described by Ludwig and Nelson.[10]

(13) J. B. Lutz and J. M. Nelson, *J. Biol. Chem.*, **107**, 169 (1934).

(14) O. Warburg and H. A. Krebs, *Biochem. Z.*, **190**, 134 (1927).

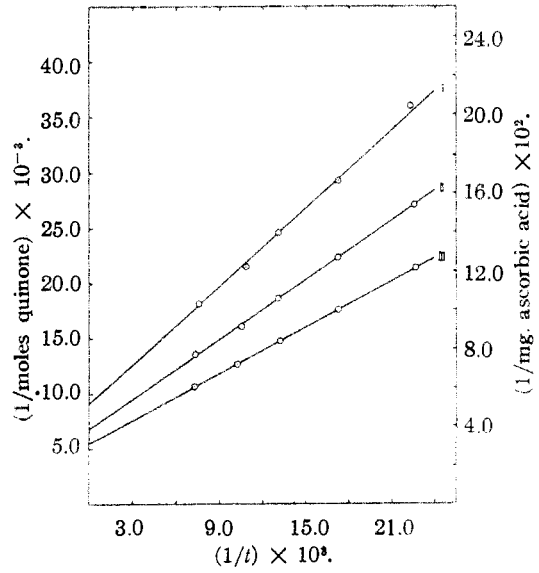


Fig. 2.—Reciprocal plot of data in Fig. 1. The linear curves (I, II and III) are expressed by Equation 2 and therefore verify that the initial reaction course during the enzymatic oxidation of catechol follows Equation 1. The most probable curve was established in each case by the method of residuals[15] using large-scale plots.

The Initial Reaction Velocity.—When the reciprocal of the quinone is plotted against the reciprocal of the time, linear curves are obtained as shown in Fig. 2. This fact indicates that during the initial stages of the enzymatic oxidation of catechol by a purified high catecholase preparation of tyrosinase the *o*-benzoquinone is produced in accordance with the following equation

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

since the linear curves are expressed by the reciprocal equation

$$1/Q = b/a(1/t) + 1/a \qquad (2)$$

where Q is the *o*-benzoquinone, and a and b are constants which may be evaluated by determining the slope and intercept of the linear curves of Eq. 2.

Differentiation of Eq. 1 in respect to t gives equation 3.

$$\frac{dQ}{dt} = \frac{ab}{(b + t)^2} \qquad (3)$$

and it follows that when $t \cong 0$

$$\frac{dQ}{dt_{(t \cong 0)}} = a/b \qquad (4)$$

Thus the initial reaction velocity (a/b) is the reciprocal of the slope of the linear curves shown in Fig. 2 (see Eq. 2) and may be determined from the experimental data.

The Unit of Catecholase Activity.—In all previous publications from this and certain other laboratories concerning the enzyme tyrosinase, one unit of enzyme activity has been defined as that amount of enzyme that, when catalyzing the oxidation of the phenolic substrate under specified conditions, causes an oxygen uptake of 10 cu. mm. per minute. This rate of oxygen uptake is equivalent to 8.93×10^{-7} mole of quinone per minute or 1.49×10^{-8} mole of quinone per second. Thus in terms of the initial reaction velocity (Eq. 4), which may be expressed as moles of quinone per second, the catecholase activity is calculated from

$$\text{Catecholase Units} = (a/b)/1.49 \times 10^{-8} \qquad (5)$$

and one unit of catecholase becomes that amount of enzyme which produces *o*-benzoquinone at an initial rate of 1.49×10^{-8} mole of quinone per second. The original definition of a catecholase unit has not been changed, but the portion of the reaction from which the unit is calculated has been shifted.

Crude and Partially Purified Enzyme Preparations.—As previously mentioned, the inactivation of the enzyme occurring during the oxidation of catechol is dependent to a certain extent on the purity of the preparation, *i. e.*, freedom from extraneous inactive protein material.[16] Thus when very crude preparations of the enzyme are employed, such as the pressed juice of the mushroom, the production of quinone may be linear with time during the initial stages of the reaction (see Fig. 3). The calculation of catecholase activity in these cases is, of course, simplified since the slope of the reaction curve is constant and gives directly the initial reaction velocity in moles of quinone per second.

It can be seen that a linear reaction course is nothing more than a special case of the expression as given in Eq. 1. For as the constants a and b become very large, *i. e.*, approach infinity, b becomes very large in respect to t and Eq. 1 approaches the expression for a straight line through the origin, *i. e.*

$$Q = at/b = kt \qquad (6)$$

As the purification of a high catecholase type preparation of tyrosinase is increased, *i. e.*, the activity per mg. dry weight is increased, the inactivation of the enzyme during the initial stages of the oxidation of catechol soon becomes evident. That is, the reaction course generally departs from a linear form after two or three steps in the purification process. It has been our experience in following through a large number of such preparations that the reaction course always follows the expression given by Eq. 1, *i. e.*, the reciprocal plot

(15) H. M. Goodwin, "Elements of the Precision of Measurements and Graphical Methods," McGraw-Hill Book Co., New York, N. Y., 1920.

(16) It should be pointed out that the inactivation characteristics of a tyrosinase preparation during the oxidation of catechol appear also to be dependent on the ratio of the catecholase to cresolase activity of the enzyme.[5] Preparations relatively high in cresolase activity generally are more stable than high catecholase preparations during the oxidation, and several purified high cresolase preparations have been obtained that show no inactivation during the initial stages of the oxidation of catechol.[5]

of quinone versus time yields linear curves of the type expressed by Eq. 2 and shown in Fig. 2. The values of a and b decrease to constant values that are characteristic of the high catecholase type preparation, and further purification has little or no effect on the curvature of the reaction course. In other words, after a certain stage in the purification is reached, further purification has little or no effect on the inactivation characteristics of the high catecholase type preparation. The application of Eq. 1 to a study of the inactivation of the catecholase activity of tyrosinase is to be published later.

The Activity Determination: Experimental.—The chronometric method as originally described made use of a 250-cc. reaction volume. Experience in this Laboratory has shown that the reaction system can be reduced to a 100-cc. volume with advantage if all of the reagents of the system are reduced proportionately, *i. e.*, to 2/5 scale. Much less enzyme, catechol, ascorbic acid and buffer are used up, therefore, per end-point determination; an important factor when a large number of determinations are made per day. The precision of the measurements and working range of enzyme concentration do not appear to be significantly different in the smaller volume. The method as described below is, therefore, for the 100-cc. volume system.

A 300-cc. round-bottom three-neck flask[17] is clamped in position in a 25° thermostat. One neck of the flask is to hold a capillary siphon tube for carrying the reaction mixture dropwise (about 2 drops per second) over into the acidified starch iodide indicator solution. Another neck allows for the introduction of a medium stream of air bubbles which serve to agitate the contents of the flask and also to start the siphon flowing when the third (center) neck is momentarily closed. The center neck of the flask is used for introducing the reagents which are previously maintained at 25° in the thermostat.[18]

In the flask are placed 10 cc. of 0.2 M citric acid−0.4 M secondary sodium phosphate buffer (pH 5.1) and enough water to make the total volume 100 cc. when the other reagents have been added. Just previous to starting the reaction the ascorbic acid and enzyme are added. Usually for the first end-point an accurately known quantity of ascorbic acid (about 3 mg.) is introduced in the form of a 0.1% solution which is also 0.1% in metaphosphoric acid (the latter stabilizes the ascorbic acid to autoxidation but has no effect on the enzymatic reaction). The ascorbic acid and enzyme are rinsed in with a measured quantity of water, and the siphon tube is then adjusted in position. The air bubbler is inserted, and the reaction is initiated about ten to fifteen seconds afterwards by rapidly introducing an aqueous solution of catechol from a small erlenmeyer flask or cylinder. The pH of this final reaction is 5.5. At any convenient time thereafter (usually 5 to 10 seconds) the siphon is started, and the end-point time, the time at which o-quinone is existent in the reaction mixture due to complete removal of reducing agent, is indicated by the appearance of a blue color at the point where the reaction mixture drops into the acidified starch-iodide indicator solution. The indicator solution is a mixture of 5 cc. of 1% starch solution, 25 cc. of 10% potassium iodide solution, and 25 cc. of 2 N sulfuric acid containing 1% pyrogallol. To greatly facilitate the observation of the end-point it is advantageous to use the indicator solution in a small crystallizing dish illuminated from below through white opal glass. The indicator solution should be stirred slowly at a constant rate during the determination. The effect on the end-point time caused by certain variations in experimental conditions has been discussed elsewhere.[2]

To determine the catecholase activity of tyrosinase by this method it is first necessary to establish the optimum concentration of substrate (catechol) to be used, since the rate of oxidation varies with the catechol concentration for different tyrosinase preparations.[2] This is best accomplished by diluting the enzyme so that when 50 mg. of catechol and 3 mg. of ascorbic acid are used an end-point is obtained in the region of forty to sixty seconds. Then using the same amount of enzyme and ascorbic acid the end-point determination is repeated three or four times while varying the original catechol concentration until the quantity giving the shortest end-point time has been determined. Purified high catecholase preparations usually have an optimum catechol concentration in the vicinity of 20 mg. per 100 cc. reaction volume, whereas crude preparations and high cresolase preparations generally show maximum activity and little variation between 100–200 mg. of catechol.

With the optimum catechol concentration established the initial reaction course is then determined by repeating

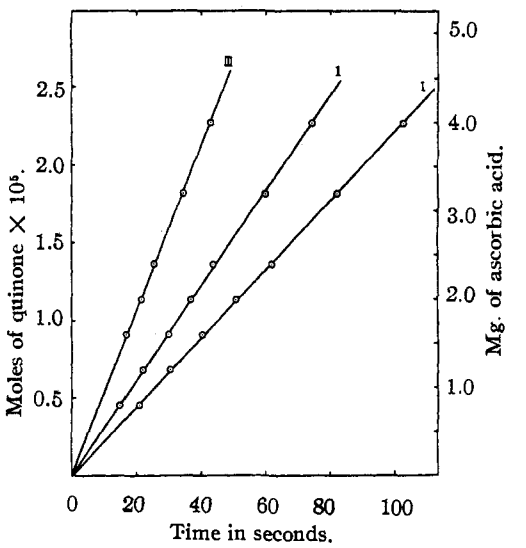


Fig. 3.—Curves showing the linear production of o-benzoquinone during the initial phase of the enzymatic oxidation of catechol when catalyzed by three different amounts of crude extract preparation[19] of tyrosinase from the common mushroom, *Psalliota campestris*. Each experimental point was obtained and checked using 100 mg. of catechol (optimum) in a 100-cc. reaction volume buffered to pH 5.5 as described in the experimental section. Curve I, 1 cc. of a 2:17 dilution of the extract; Curve II, 1 cc. of a 3:18 dilution; Curve III, 1 cc. of a 5:17 dilution. See Table I for calculation of catecholase activities. The undiluted crude extract had a dry wt. of about 6 mg./cc. and contained 23 cresolase units/cc.

(17) A 500-cc. flask works equally well.

(18) The diluted enzyme is kept at 0–5° at all times. Experience has shown that many tyrosinase preparations, particularly when purified, tend to lose catecholase activity very rapidly when allowed to stand in highly dilute solution at room temperature. Concentrated preparations (in excess of 1 mg. of enzyme protein per cc.) are generally quite stable. To dilute the stock enzyme for activity determination cold water should be used, and the analysis made within a few minutes of the dilution. Several determinations generally can be made on the same dilution if it is kept in an ice-bath at 0–5°.

(19) 1 lb. of mushrooms was passed through a meat grinder into two volumes of cold acetone (chilled with dry-ice) to wash out natural substrates and coloring matter. After filtering immediately, the pulp was frozen with dry-ice for two hours and then broken up and suspended in 600 cc. of water containing a drop of concentrated ammonia solution. After standing overnight at refrigerator temperature the extract and pulp were separated by pressing through drain-cloth.

the end-point determination a number of times using the same amount of enzyme and catechol but varying the amount of ascorbic acid used—usually from 1 to 4 mg. so that the end-points lie in the range of twenty to one hundred seconds. Usually three or four such end-points (with checks) are sufficient to determine the reaction course as plotted in Fig. 1 or 3.

Calculation of Catecholase Activity from Data.—If no inactivation of the enzyme is apparent, as in the case of the data as shown in Fig. 3, the initial reaction velocity is given by the slope of the reaction curve (see Eq. 6), and the catecholase activity is obtained directly by dividing the slope in moles of quinone per second by 1.49×10^{-8}. If the slope is obtained in mg. of ascorbic acid per second, it should be divided by 2.62×10^{-3} to give units of catecholase involved (see Table I).

TABLE I

CALCULATION OF CATECHOLASE ACTIVITY FROM DATA. NO ENZYME INACTIVATION APPARENT. DATA OF FIG. 3

Curve	Enzyme diln. factor	Slope, mg. AH_1 per sec.	Units in reaction, slope/ 2.62×10^{-3}	Units/cc., undiluted
I	8.5	0.0388	14.8	126
II	6.0	.0540	20.6	124
III	3.4	.0933	35.6	121
			Average ± a.d.	124 ± 2

If inactivation of the enzyme is apparent, as in the case of the curves in Fig. 1, the best value of the catecholase activity is obtained from the reciprocal plot of the data as shown in Fig. 2. If care is taken to establish the best linear curve through the experimental points (see legend Fig. 2) the reciprocal of the slope of this curve (the initial reaction velocity) is directly proportional to the amount of enzyme taken when the conditions specified above are employed. This proportionality is shown by the data as arranged in Table II.

TABLE II

CATECHOLASE ACTIVITY FROM THE RECIPROCAL PLOT DATA (FIG. 2). ENZYME INACTIVATION APPARENT

Curve	Enzyme, cc.	Slope (b/a), sec. per mole Q	Initial vel. (a/b) moles Q per sec.	Catecholase units $(a/b)/1.49 \times 10^{-8}$	Units/cc.
I	6.0	11.8×10^5	8.48×10^{-7}	56.8	9.47
II	8.0	8.94×10^5	11.2×10^{-7}	75.2	9.40
III	10.0	7.03×10^5	14.2×10^{-7}	95.4	9.54
				Average ± a.d.	9.47 ± 0.05

If only the activity of the enzyme is desired and not the values of a and b of Equation 1 or 2, it is not necessary to plot the reciprocal curve (Fig. 2). The initial reaction velocity can be obtained directly from the original data by inserting the values of two experimental points into the following equation

$$a/b = \frac{(Q_1Q_2)(t_2 - t_1)}{(t_1t_2)(Q_2 - Q_1)} \qquad (7)$$

where Q_1 is the moles of quinone formed in t_1 seconds, and Q_2 the moles formed in a longer time t_2. It is generally more convenient to use Eq. 7 in terms of mg. of ascorbic acid rather than moles of quinone, since the data are obtained directly in terms of ascorbic acid. The initial reaction velocity is then converted into catecholase activity in the manner described previously and illustrated in Table I. This method of calculating the activity directly from the original data is much less time-consuming than the method involving the reciprocal plot. However, the results obtained are less reliable since they are based on only two experimental points. For example, using this method for three different pairs of points (1 and 3, 2 and 4, 3 and 5) for each of the three curves of Fig. 1 yields an average value of 9.37 ± 0.33 (3.5%) units per cc. as the activity of the diluted enzyme (compare with Table II).

Discussion

It seems advisable to compare the catecholase activity of tyrosinase as determined by the method described above with that obtained by other methods of determination. Two other methods that are in use are (I) the direct monometric method on catechol,[3,20] and (II) the manometric method using a mixture of hydroquinone and catechol as proposed by Adams and Nelson.[2] Each of these methods (I and II) depends on a rate measurement made at some time after the enzymatic oxidation of catechol has been initiated and the activity results are, therefore, influenced by what has happened to the enzyme during the reaction previous to that rate measurement. It follows, therefore, that only in the case of an enzyme which shows no inactivation during the initial stages of the reaction can these methods yield results that are comparable to the results obtained with the chronometric method as described above. In the usual case, where inactivation of the enzyme is evident, the chronometric method based on the initial reaction velocity will give considerably higher values of catecholase activity. It follows that no conversion factor is possible to convert catecholase activity as determined by any of these methods into activity as based on the initial reaction velocity unless the nature and degree of inactivation in the early stages of the reaction are also known.

In the direct manometric method on catechol, as used by Gregg and Nelson,[3] the inert protein material gelatin is placed in the reaction system to "protect" the enzyme to some extent against inactivation. More reproducible results are obtained in the presence of gelatin than when gelatin is absent from the system, and the reaction course is somewhat more linear in form during the first two minutes of the reaction when gelatin is employed.[6] The diluted enzyme stands in the buffered reaction system (containing gelatin) during a ten to twenty minute temperature equilibra-

(20) D. Keilin and T. Mann, *Proc. Roy. Soc.* (London), **125B**, 187 (1938).

(21) M. H. Adams and J. M. Nelson, THIS JOURNAL, **60**, 2474 (1938).

tion period (25°), and then the reaction is initiated by the addition of catechol. Readings of oxygen uptake are taken every minute.

If the activity of a purified high catecholase preparation of tyrosinase is determined by this method and then by the chronometric method as based on the initial reaction velocity, about twice as much catecholase activity will be indicated in the latter case. A good share of this discrepancy between the two methods is due to the considerable inactivation of the enzyme that occurs during the first minute of the reaction, and the rest can undoubtedly be ascribed to the difference in the experimental conditions. The main experimental difference between the two systems lies in the fact that in the chronometric system no gelatin is employed, and the diluted enzyme is not subjected to a long temperature equilibration period prior to the measurement. Both of these factors have been shown to lower the activity of the enzyme, particularly when the activity is based on the first minute reading of oxygen uptake.[6]

The Adams and Nelson manometric method of measuring the catecholase activity of tyrosinase[21] is based on the rate of oxygen absorption while the enzyme catalyzes the oxidation of a small amount of catechol in the presence of a relatively large amount of hydroquinone. Gelatin is also employed in the system. At the time it was proposed, it was believed that the hydroquinone functioned only as a reductant of the *o*-benzoquinone, thereby maintaining a constant concentration of catechol during the measurement. The presence of hydroquinone in the catechol–enzyme system markedly stabilizes the enzyme against inactivation during the oxidation, and the reaction course as followed by oxygen uptake tends to be linear for an appreciable time.[10] Because of the linear reaction course, reproducible rate measurements of the enzymatic oxidation of catechol became possible with the introduction of this substrate mixture, and the method met with favor. It did much to stimulate studies of the catecholase activity of tyrosinase, and made possible the comparison of this activity with the monophenolase activity of the enzyme.

However, these studies have shown that hydroquinone does not act merely as a reductant for the *o*-benzoquinone. Under certain conditions it is oxidized by the enzyme,[22] and previous publications[4,6] have emphasized the fact that the stabilizing effect of hydroquinone against the inactivation of the enzyme varies with the nature of the enzyme preparation. In all cases when hydroquinone is in the system the initial rate of enzymatic oxidation is less than when catechol is oxidized alone, *i. e.*, catecholase activity obtained by this method is always less than that obtained in the direct manometric method on catechol. Apparently the hydroquinone behaves as a competitive inhibitor in the system.

If these effects of hydroquinone were constant, no serious criticism to the continued use of the method could be offered but, as a matter of fact, both the stabilizing effect and the degree of inhibition vary widely with factors such as the purity of the enzyme and the ratio of the catecholase and cresolase activities. It is obvious that the catechol–hydroquinone method does not give a true measure of the catecholase content of any tyrosinase preparation, and the continued use of the method is, therefore, open to serious criticism.

Summary

1. A chronometric method based on the indirect titration of *o*-benzoquinone is described for following the initial reaction course during the enzymatic oxidation of catechol.

2. The precision is such that the initial reaction velocity (dQ/dt at $t \cong 0$) may be determined and used for calculation of the catecholase activity of the enzyme tyrosinase.

3. The value of catecholase activities based on the initial reaction velocity, particularly when comparing enzymes having different inactivation characteristics, has been discussed.

4. Two other methods now in use for the measurement of tyrosinase catecholase activity have been discussed in comparison with the chronometric method.

5. It is felt that the chronometric method of measuring the catecholase activity of tyrosinase is superior to other methods now in use.

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(22) D. C. Gregg and J. M. Nelson, THIS JOURNAL, **62**, 2510 (1940).