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A New Method for the Measurement of Tyrosinase Catecholase Activity

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Various investigators[1,2,3] have observed that the ratio of catecholase activity to cresolase activity[4] in a given amount of the enzyme tyrosinase is not necessarily a constant factor. During the process of isolation and purification of the enzyme the ratio of these activities can be varied almost at will within certain limits. This observation has resulted in considerable effort being directed toward determining whether or not the enzyme is in reality one protein possessing two types of enzymic activity or is a mixture of two protein enzymes each with its own activity.[2,3,5]

On attempting to apply electrophoretic methods to this problem it soon became apparent that the methods in use for the determination of catecholase activity are very unsatisfactory. Most of the methods are based on oxygen absorption measurements. The action of the enzyme with catechol as a substrate differs markedly in several respects from its action with *p*-cresol as a substrate when a comparison is made on the basis of these measurements. In the former case the enzyme experiences such rapid inactivation that rate measurements have little significance for the calculation of enzyme activity unless they are made within the first two minutes. The experimental error involved in making such a rate measurement in a respirometer is very considerable and has been estimated by Parkinson and Nelson[2] to be about 20–30%.

TABLE I[a]

SHOWING THE REPRODUCIBILITY OF THE MANOMETRIC RATE METHOD FOR THE DETERMINATION OF CATECHOLASE ACTIVITY USING CATECHOL AS THE SUBSTRATE

Prepn.	Consecutive determinations	Mean activity units in flask	Range of measurements	a.d. $\epsilon d/u$	a.d. %	A.D. a.d./$\sqrt{\mu}$	A.D. %
High	18	2.29	1.93–2.64	±0.20	± 8.7	±0.047	± 2.1
Catecholase[6]	2	2.27	1.95–2.58	±0.32	±14.1	±0.23	±10.1
High	15	2.31	1.93–2.64	±0.21	± 9.1	±0.054	± 2.3
Cresolase[6]	2	2.29	1.93–2.64	±0.35	±15.3	±0.25	±10.9

[a] Barcroft differential respirometers[7] were used in determining the activities. The reaction flasks were of 50-cc. capacity, temp. 25°. Reaction mixtures contained 1 cc. of 0.2 *M* citrate–0.4 *M* phosphate buffer (*p*H of reaction mixture 7.1); 1 cc. of aqueous solution of gelatin (5 mg.); enzyme solution containing about 2.30 catecholase units, 1 cc. of an aqueous solution of catechol (4 mg.) added from the side arm at zero time, and sufficient water to give a total reaction volume of 8 cc. At zero time the speed of shaking of the manometers was increased from 60 to 160 oscillations per minute. Readings were taken every minute for the first five minutes but due to the rapid inactivation only the first two readings were used to calculate activities. The two determinations indicated above for each preparation are the two most widely discrepant consecutively run values obtained during the larger number of determinations.

A careful study of the reproducibility of this method for determining activity using two different types of enzyme preparations shows that the range or maximum difference in activity to be expected in a series of determinations is of the order of 20–30% of the mean value. The data in part are summarized in Table I. From the last column in the table it can be seen that with 15–18 determinations it is possible to obtain a mean activity reliable to about ±2–3%. However, if only two consecutive measurements are made, the reliability may be decreased by five times this value. The good agreement between the mean of two determinations and the mean value obtained with the larger number is fortuitous and due to the particular choice of data to illustrate the range. This agreement cannot therefore be considered as an indication of the reproducibility of the mano-

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

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

(3) D. Keilin and T. Mann, *Proc. Roy. Soc.* (London), **B125**, 187 (1938).

(4) One unit of catecholase is defined as the amount of enzyme required to cause the uptake of 10 cu. mm. of oxygen per minute when acting on 4 mg. of catechol. One unit of cresolase is the amount of enzyme required to cause the uptake of 10 cu. mm. of oxygen per minute when acting on 4 mg. of *p*-cresol.

(5) D. C. Gregg and J. M. Nelson, THIS JOURNAL, **62**, 2500 (1940).

(6) A high catecholase preparation has been defined[2,5] as any enzyme preparation in which the catecholase activity is proportional to the copper content and the ratio of catecholase to cresolase activity is high (more than 2). A high cresolase preparation has been defined as any enzyme preparation in which both the catecholase and cresolase activities are proportional to the copper content and the ratio of catecholase to cresolase is low (less than 2).

(7) M. Dixon, "Manometric Methods," University Press, Cambridge, 1934.

metric method. To use this method, then, a large number of determinations are necessary to obtain a reliable value of catecholase activity. This degree of reliability of the mean, and particularly the time required to obtain it, constitute a serious handicap to the problem of differentiating between the two activities of the tyrosinase preparations.

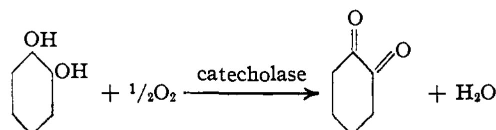
A method involving the use of a mixture of hydroquinone and small amounts of catechol as a substrate has been suggested by Adams and Nelson.[1] A much higher degree of precision is possible with this method for the hydroquinone appears to exert a protecting effect on the enzyme and the rate of oxygen absorption has been reported to be constant for an appreciable period of time. However, this method of measuring catecholase activity is now open to serious criticism. Gregg and Nelson[8] have demonstrated that the hydroquinone is enzymatically oxidized by enzyme preparations that are relatively high in cresolase activity. It is very doubtful whether a true measure of catecholase activity is obtained even with enzyme preparations relatively low in cresolase activity for the hydroquinone materially lowers the rate below that obtained with catechol alone and thus does not function only as a simple reducing agent as at first supposed.

The inactivation of the enzyme during the oxidation of catechol is so striking that it has been suggested by Ludwig and Nelson[9] as a basis for the measurement of catecholase activity. For any given enzyme preparation the total amount of oxygen absorbed during the complete inactivation of a given amount of the enzyme is proportional to the amount used. The proportionality factor is approximately constant for all high catecholase preparations and high cresolase preparations, but for the borderline or intermediate preparations, the proportionality factor fluctuates seriously from one enzyme preparation to another.[10]

From the preceding discussion it will be apparent that a rapid, reproducible, and relatively precise method for the determination of catecholase activity will greatly facilitate the solution of the problem of differentiating and characterizing the catecholase and cresolase activities of the enzyme tyrosinase. It is the purpose of this communication to report the development of such a method.

(8) D. C. Gregg and J. M. Nelson, THIS JOURNAL, **62**, 2510 (1940).
(9) B. J. Ludwig and J. M. Nelson, *ibid.*, **61**, 2601 (1939).
(10) W. H. Miller and C. R. Dawson, THIS JOURNAL, **63**, 3368 (1941).

As previously mentioned, most of the methods available for the determination of catecholase activity depend on oxygen absorption measurements. It has been demonstrated[11] that after the initial production of *o*-benzoquinone during the enzymatic oxidation of catechol, the mechanism of the oxidation reaction becomes complex and probably varies with conditions of *p*H and catechol concentration as well as with concentration of enzyme. For this reason it is not at all certain that rate measurements of oxygen absorption during the enzymatic oxidation of catechol are a true measure of the rate of conversion of catechol to *o*-benzoquinone, except possibly when conditions are controlled so as to minimize the secondary reactions. Since the reaction



is the real catecholase reaction by definition, it seems desirable to base a new method for the measurement of catecholase activity on the rate of formation of *o*-benzoquinone.

The method reported here makes use of the principle of keeping the *o*-benzoquinone continuously in the reduced state, *i. e.*, as catechol, during the short period of time the enzyme is being measured, thereby eliminating the possibility of complicatings econdary reactions involving the quinone. This is accomplished by allowingt he enzyme to catalyze the oxidation of catechol in the presence of a limited but definite amount of reducing agent. This agent enables the catechol to function as a shuttle and thus its concentration remains constant until the reducing agent is completely oxidized. Obviously the reducing agent is a substance which is not oxidized by the enzyme nor does it in any measurable way inhibit or accelerate the enzyme action. As soon as the reducing agent has been completely oxidized, *o*-benzoquinone appears in the solution and can be detected by suitable means. The determination of enzyme activity therefore becomes a measurement of the time required for a certain quantity of enzyme to produce enough *o*-benzoquinone to oxidize completely a definite quantity of reducing agent. Since the method is based on a measurement of time, for ease of discussion and comparison it will be referred to as the chronometric method.

(11) C. R. Dawson and J. M. Nelson, *ibid.*, **60**, 245–256 (1938).

In practice, ascorbic acid has been found to be a satisfactory reducing agent. The appearance of *o*-benzoquinone in the enzyme reaction vessel is detected with good sensitivity by continuously sampling the solution dropwise into an acidified solution of potassium iodide containing starch. The end-point is indicated by the first appearance of a blue color. This technique permits the use of relatively high concentrations of catechol, and solutions buffered to *p*H values for optimum enzyme activity. Furthermore, the enzyme activity can be measured with good precision within the first minute or so of its action, thereby allowing good estimations of the initial reaction velocity. In view of the effect of environmental factors on the course of the enzyme action[10] this is a distinct advantage over existing methods for the determination of activity.

Analytical Procedure

The method in detail is as follows. A 500-cc. round-bottom three-neck flask is clamped in position in a 25° thermostat. The reagents to be added are maintained at 25°. In the flask are placed 25 cc. of 0.2 *M* citrate–0.4 *M* phosphate buffer (*p*H 5.1) and enough water to make a total volume of 250 cc. when the other ingredients have been added. The *p*H of the reaction mixture is 5.5. Just previous to starting the actual determination, 8 mg. of ascorbic acid in about 8 cc. of a 0.1% metaphosphoric acid solution and the enzyme solution are added. These solutions are rinsed in with a measured quantity of water and the siphon tube for sampling the contents of the flask is then adjusted in a side neck. Into the other side neck about fifteen seconds before zero time is placed a tube through which passes a medium stream of air which serves to agitate the contents of the flask. At zero time, a solution of catechol in 25 cc. of water is rapidly introduced from an Erlenmeyer flask, and fifteen to twenty seconds later this neck is momentarily closed with a rubber stopper until the air pressure within the flask has started the siphon. The solution siphons dropwise into a crystallizing dish (about 10 cm. in diameter) containing 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. In order to facilitate the observation of the end-point the crystallizing dish is illuminated from below through white opal glass and its contents are stirred with a small motor-driven stirrer. The siphon tube is made from thick-walled capillary glass tubing of about 1 mm. bore and is so constricted that about two drops per second or approximately 12–13 cc. per minute pass through it. The end-point or the time at which *o*-benzoquinone is existent in the solution due to lack of reducing agent, is indicated by the appearance of a definite blue color at the point where the reaction mixture drops into the starch–acid mixture. The time between the appearance of the first trace of color and a result apparent to an inexperienced eye is not over a second in the favorable ranges of enzyme concentration.

To calibrate the chronometric method against the manometric method it is necessary to compare the behavior of known quantities of enzyme preparations using both methods over the same period of time. In this way, any discrepancy which might arise due to the rapid inactivation of the enzyme should be at a minimum. Since observations manometrically are taken only every minute, a quantity of enzyme was chosen such that the end-point in the new chronometric method would be observed in the immediate vicinity of sixty seconds. The results obtained with four different enzyme preparations are summarized in Table II. In order that results with the new method may be compared easily with those obtained in the past using the manometric method, a calibration value of 34 catecholase units has been selected as that amount of enzyme giving an end-point of sixty seconds in the chronometric method. By comparing columns 7 and 9 of this table it will be seen that on this basis, a reasonably good correlation exists between the two methods. No better correlation is to be expected because of the nature of the precision of the manometric method. This difference in order of reliability of results obtained by the two methods is clearly seen by comparing columns 8 and 10 and 3 and 4 in the table. Only a few determinations with the rapid chronometric method are necessary to obtain a result several times as reliable as that obtained from a large number of observations using the slower manometric method.

To determine enzyme activity by the new method it is not necessary to adjust the enzyme concentration until an end-point of exactly sixty seconds is obtained. By using relatively larger or smaller quantities of enzyme, the end-point is obtained in less or more than sixty seconds. However, due to the inactivation of the enzyme, which becomes more pronounced as the end-point time increases, the relationship between end-point time and quantity of enzyme used is not linear. This is shown in Fig. 1, where curve I represents the data obtained with a high catecholase preparation and curve II that obtained with a high cresolase preparation. The curves intersect at the one minute point since they are both plotted on the basis that an end-point of sixty seconds is equivalent to 34 catecholase units. It is apparent that the inactivation characteristics of high catecholase and high cresolase preparations are different, and these differences become evi-

TABLE II[a]

SHOWING THE CORRELATION OF THE MANOMETRIC AND CHRONOMETRIC METHODS FOR THE DETERMINATION OF CATECHOLASE ACTIVITY AT pH 5.5

Number	Enzyme preparation, kind	Experiments Mano-metric	Chrono-metric	End-point time, Chronometric Seconds	A.D.	Enzyme activity Manometric Units	% A. D.	Chronometric Units	% A. D.
C143F1	High catecholase	18	15	56.8	±0.23	34.7	±2.0	35.9	±0.40
C144F1B	High catecholase	9	3	57.8	.35	33.9	±3.8	35.3	± .61
C143F2	High cresolase	15	4	60.4	.35	35.8	±2.4	33.9	± .58
C143F3	High cresolase	18	3	59.0	.17	34.6	±2.7	34.6	± .29

[a] The chronometric units (column 9) are calculated on the basis that 34 catecholase units give an end-point of sixty seconds (see calibration curves, Fig. 1) when 8 mg. of ascorbic acid is used as reducing agent according to the procedure described in this paper: 50 mg. of catechol was used with the high catecholase preparations and 150 mg. with the high cresolase preparations. The manometric data were obtained as described in the legend of Table I except that the pH of the reaction mixture was 5.5, no gelatin was used, and the enzyme rather than the substrate was added from the side arm at zero time. For high catecholase preparations, manometric rates using this procedure are the same, within experimental error, as those obtained at pH 7.1 with or without gelatin.[10] For high cresolase preparations the change in pH from 7.1 to 5.5 in the absence of gelatin is without effect, but in the presence of gelatin causes a 20–30% decrease in rate. All tyrosinase preparations used in these studies were prepared from the common mushroom, *Psalliota campestris*, according to the methods reported from these Laboratories.[2,9] Data characterizing the above preparations are summarized as follows:

Preparation	C143F1	C144F1B	C143F2	C143F3
Catecholase units (chronometric) / Cresolase units	9.5	13.4	1.50	1.80
Copper, %	0.07	0.10	0.04	0.06
Catecholase units per γ copper	1100	965	460	480

The method for the determination of cresolase activity was that described by Gregg and Nelson.[5] The method for the determination of copper was the same as that used by Parkinson and Nelson.[2]

dent within the first minute of enzyme action. Before secondary factors such as surface coagulation and salt concentration can exert any significant effect on the rate of inactivation, the shape of these curves is that of a rectangular hyperbola. Thus when the reciprocal of the end-point time is plotted against quantity of enzyme, a linear relationship is found (see curves III and IV). The equations of these straight lines give expressions which can be used directly for the calculation of enzyme activity. They are, for the

High catecholase prepn. (Curve III):

$$\text{Units} = (1440/t) + 10$$

High cresolase prepn. (Curve IV):

$$\text{Units} = (1830/t) + 3.8$$

With calibration curves of this type or the expressions obtained from them, it is possible to determine catecholase activity from an end-point occurring anywhere between thirty and 180 seconds provided it is known whether the enzyme preparation is high catecholase or high cresolase in character. An easy and striking way to characterize an enzyme preparation is to observe its behavior relative to different quantities of catechol. The difference between high catecholase and high cresolase preparations in this respect is quite marked as can be seen in Table III. Data for an intermediate preparation are also included in this table.[12] It is readily seen that the amount of substrate (150 mg.) which gives a maximum rate with a high cresolase preparation exerts a definite restrictive action on a high catecholase preparation. The latter has a maximum rate with 50 mg. of catechol which is insufficient substrate to give a maximum rate with the other two preparations;

TABLE III[a]

SHOWING THE DIFFERENT EFFECTS OF CATECHOL CONCENTRATIONS ON THE ENZYME ACTIVITY OF THREE DIFFERENT TYPES OF TYROSINASE PREPARATIONS

Substrate, mg./250 cc.	25	50	100	150	200	250
High catecholase	97	100	88	80		
Intermediate		94	100	100	98	93
High cresolase		84	95	100	99	98

[a] The data in the table are given as per cent. of the maximum observed rate for the preparation considered. The procedure and the composition of the reaction mixtures used were as outlined for the new chronometric method. The quantity of enzyme used was chosen so that when it functioned at maximum rate it gave an end-point at about sixty seconds. The only variation then in the reaction mixture was the catechol concentration. Similar results were obtained with six other high catecholase, two other intermediate and one other high cresolase preparations, and this type of characterization was verified in all cases by subsequent studies.

(12) The authors have observed that enzyme preparations are occasionally obtained having properties similar in some respects to each of the above types. Such preparations have been designated as intermediate preparations.[10]

250 mg., which exerts practically no restrictive action on a high cresolase preparation, noticeably restricts the activity of an intermediate preparation. Thus the behavior of the intermediate preparation in this respect lies in between that of the other two types. These studies suffice to show that in working with an unknown enzyme preparation, it is first necessary to ascertain the correct substrate concentration which gives the maximum enzyme activity. Such a study will characterize the preparation and allow the determination of enzyme activity over a considerable end-point range using calibration curves of the type of Fig. 1. It is noted in this figure that there is no curve for the intermediate preparations. These preparations, unlike those of the other two types, vary enough in their properties so that no one curve indicates the behavior of the group. However, these curves, when plotted, are all in between those of the high catecholase and high cresolase preparations, their position being dependent on the extent to which they resemble either of these two main types. That curve I describes the behavior of high catecholase preparations is indicated by the fact that it was followed over a range from about thirty to 150 seconds within experimental error by seven such preparations. The high cresolase curve described exactly the behavior of two high cresolase preparations. Over a time range from forty-five to ninety seconds the maximum spread between the curves intersecting at sixty seconds approaches 5–6%. For a more narrow range from fifty to eighty seconds, the spread approaches 3.5–3.7%. Thus either curve, regardless of kind of tyrosinase preparation may be used for the determination of enzyme activity over a limited range in the vicinity of sixty seconds.

Discussion

The End-point.—The starch–hydriodic acid method for detecting the end-point proved to be the most satisfactory of several methods tried. Concentrations of potassium iodide greater than 10% do not increase the reproducibility of the end-point but tend to obscure it because of shadow effects caused by density differences in the crystallizing dish. For the same reason the concentration of sulfuric acid used was fixed at 2 *N*. It is important that the concentration of the acid in the crystallizing dish at all times be such as to immediately and completely inactivate the enzyme, thus eliminating the possibility of quinone production in the acid mixture. The 1% of pyrogallol tends to prevent aerobic oxidation of the hydriodic acid solution and does not appear to otherwise influence the end-point in any way. Thus, with the pyrogallol present, the starch–hydriodic acid mixture will remain colorless for at least fifteen minutes, while without the pyrogallol, a blue scum is noted on the surface of the solution in two to four minutes. Sulfuric acid (2 *N*) 1% in pyrogallol can be made up and kept as a stock solution.

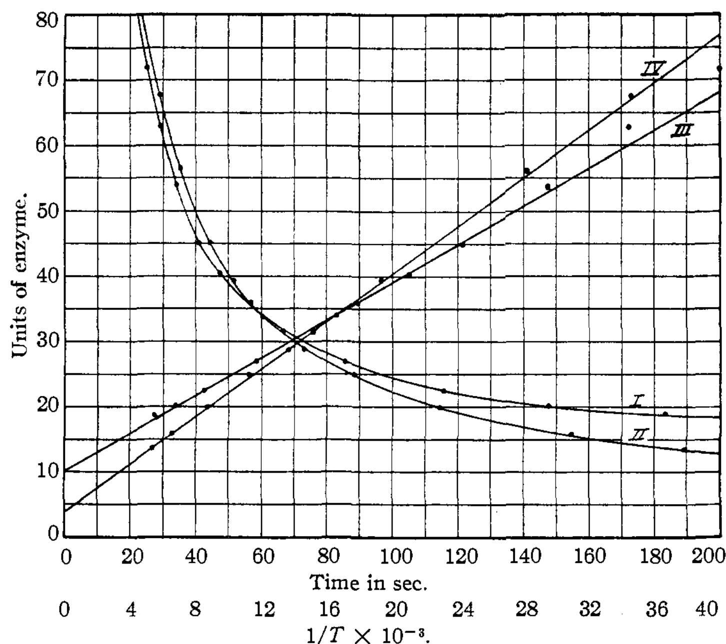


Fig. 1.—Showing the relationship between quantity of enzyme and end-point time in the chronometric method. Experimental procedure and composition of reaction mixtures as in the legend of Table II. The quantities of enzyme indicated are calculated on the basis of effective manometric catecholase activity at sixty seconds. Curve I indicates the behavior of a high catecholase preparation C143F1. Curve II is for a high cresolase preparation C143F2. Curves III and IV are obtained by plotting units *vs.* the reciprocal of time and correspond to curves I and II, respectively.

Other factors which might affect not only the sharpness but cause a delay in the end-point are rate of stirring and the presence of excess substrate or reducing agent in the crystallizing dish. The siphon tube is so arranged that the drops from

the enzyme reaction mixture fall into the crystallizing dish about midway between the periphery of the stirrer and the side of the dish. The rate of stirring in the acid mixture can be varied over a wide range without any noticeable effect on the end-point. A medium rate of about 250 r. p. m. has been found satisfactory. During the course of the reaction catechol and ascorbic acid enter the acid mixture. The authors have found that ascorbic acid has no significant effect on the end-point except when present in amounts far in excess of those possible under experimental conditions. The addition of 4 mg. of ascorbic acid to the acid mixture caused a 2–3% effect on the end-point observed at sixty seconds and a 5–7% effect on the end-point at ninety seconds. Catechol when added in quantities up to 100 mg. exerted no noticeable effect.

The Reaction Mixture.—The conditions of quantity, concentration and order of addition of the reactants warrant further discussion. It has been demonstrated that the enzyme standing in the diluted condition especially in the presence of buffer is inactivated.[10] However, the quantitative addition of the enzyme cannot be accomplished instantaneously and since the concentration of the substrate catechol is not very critical less error is involved in adding it to initiate the reaction. The enzyme therefore should be added just before zero time.

The choice of the reaction volume (250 cc.) was made after experiments showed that the range of enzyme concentration over which the activity could be effectively determined by the chronometric method was less in smaller volumes.

In most of the previous work done in these laboratories, a citrate–phosphate buffer mixture has been used since it is effective over a wide *p*H range. For activity measurements, the reactions have been buffered to about *p*H 7.1. In the chronometric method the *p*H of the reaction has been lowered to *p*H 5.5. The change to a lower *p*H has been made since the end-point appears to be sharper over a wider range of enzyme concentration and the possibility of complicating secondary reactions involving the quinone appears less. The behavior of an enzyme system in three different buffer systems of the same concentration is indicated in Table IV. It is seen that the same results are obtained over a range of thirty to 150 seconds for all three systems. Thus the inactivation of the enzyme appears the same in these three systems. The buffer concentration is not a critical one and the same results have been obtained in this method when it was varied over a considerable concentration range.

TABLE IV[a]

SHOWING THE BEHAVIOR OF THE ENZYME IN DIFFERENT BUFFER SYSTEMS

Units of prepn. C144F1B, high catecholase	End-point time in seconds		
	0.2 *M* citrate– 0.4 *M* phosphate mixture	0.2 *M* $NaC_2H_3O_2$–$HC_2H_3O_2$ mixture	0.2 *M* KH_2PO_4–Na_2HPO_4 mixture
61.6	30.0	30.3	30.2
43.1	45.7	46.1	45.0
34.6	59.0	58.4	59.0
28.1	80.0	83.7	80.3
20.5	149.0	154.1	149.0

[a] Reaction mixtures and experimental procedure as described for the chronometric method: 25 cc. of indicated buffer mixture and 50 mg. of catechol used in all cases. Final *p*H of all reaction mixtures was 5.45–5.50.

After many experiments, the choice of a reducing agent narrowed to the use of either ascorbic acid or *d*-iminoascorbic acid. Others tried and used with some success were sodium benzene sulfinate and sodium thiosulfate. It is essential that the reducing agent be easily obtained in the pure condition and remain stable in solution since the concentration of the reducing agent must be known accurately in order to determine the enzyme activity. Although ascorbic acid and *d*-iminoascorbic acid can be used interchangeably in this method, the former is much more easily obtained as a reactant of good chemical purity.

Stock solutions of ascorbic acid (usually 1 mg./cc.) can be stabilized if made up with glass distilled water containing 0.1% metaphosphoric acid. Such solutions have been found by iodimetric titration to lose only 2–4% ascorbic acid over a period of eighty days if kept refrigerated. A test of the stability of the ascorbic acid under the experimental conditions was made by bubbling air vigorously through the reaction mixture containing all but the enzyme. After a period of five minutes of bubbling, 96% of the original ascorbic acid remained as indicated by the chronometric method. That metaphosphoric acid exerts no inhibitory effect on the enzyme is shown by the fact that the same results well within experimental error were obtained whether the ascorbic acid solution contained 1%, 0.1% or no metaphosphoric acid.

With 8 mg. of ascorbic acid in a 250-cc. reaction volume the amount of oxygen available to the reaction does not become rate limiting over the

range of enzyme concentrations indicated in Fig. 1. The greatest danger of oxygen limitation is in the region where the oxygen is removed from the system by the enzyme at the greatest rate, that is, with relatively large amounts of enzyme giving end-points in the vicinity of thirty seconds. Using 12–16 mg. of ascorbic acid and correspondingly one and one-half to two times as much enzyme, the same end-point (about thirty seconds) within experimental error was observed as with 8 mg. of ascorbic acid.

That ascorbic acid functions only as a simple reducing agent and does not significantly inhibit or accelerate the enzyme action has been found to be the case. This was accomplished by titrating according to the method of Dawson and Nelson[11] the *o*-benzoquinone produced in the absence of ascorbic acid using the conditions of the chronometric method. The amount of *o*-benzoquinone formed in one minute using both high catecholase and high cresolase preparations was found to agree within 2% of that calculated on the basis of the disappearance of 8 mg. of ascorbic acid in sixty seconds. These results confirm the manometric observations of Ludwig and Nelson[9] and Sulloway (unpublished).

The rate of flow of air is not critical and may be varied to a considerable extent. If the air flow is too slow, difficulty may be experienced in starting the siphon in time to observe a quick end-point. If the air flow is too rapid, increased inactivation of the enzyme is observed in the region of 150–180 seconds for high catecholase preparations.

Highly purified tyrosinase preparations of the dilution used in these studies on standing at room temperature are not very stable. For this reason, the enzyme solutions to be measured should not stand in the reaction mixture and should be freshly prepared just before measurement by dilution from a more concentrated solution kept at all times in the refrigerator.

Studies at *p*H 7.1.—The chronometric method is based on the condition that ascorbic acid effectively reduces the *o*-benzoquinone as rapidly as formed at *p*H 5.5. Evidence supporting this view has been discussed above. Further evidence was obtained by making chronometric measurements at *p*H 7.1. Using both a high catecholase and a high cresolase preparation, the data shown in Fig. 1 were duplicated within experimental error in the favorable range of enzyme concentrations (end-points from thirty to one hundred twenty seconds). End-points occurring later than this were less reproducible at *p*H 7.1 than at *p*H 5.5. Substrate concentration studies made at *p*H 7.1 gave the same results as found at *p*H 5.5 (see Table II). These results, especially those involving the larger quantities of catechol, deomonstrate that ascorbic acid is acting as an effective reducing agent even at *p*H 7.1 where the *o*-benzoquinone is much more labile and more susceptible to secondary reactions involving catechol[11] and therefore might conceivably escape reduction by the ascorbic acid.

Comparison of the Methods.—The fundamental difference between the manometric and the new chronometric method is that the former depends on the rate of oxygen absorption while the latter depends on the rate of *o*-benzoquinone production. The changes in experimental procedure such as *p*H, buffer concentration, and the elimination of gelatin are the result of previous manometric studies on the effect of these factors on enzyme activity.[10] As regards the effect of gelatin in the chronometric method, a further reason for eliminating it is that when it is present in quantity comparable to that used in the manometric reaction mixture, the observed end-point or measure of enzyme activity becomes a function of the rate of air flow through the reaction mixture.

A distinct advantage of the chronometric method is that it operates with a high degree of reproducibility much nearer to zero time than is possible with the manometric method. As indicated in the legend of Table I, the maximum manometric rate for the catechol reaction is observed usually the first or second minute. A unit of enzyme[4] then becomes that amount of enzyme which during the first sixty seconds or between sixty and 120 seconds causes the uptake of 10 cu. mm. of oxygen. This definition of the enzyme's activity then involves an observation of the total effect of the enzyme during the given period but is not able to point out the manner of behavior during that period. The chronometric method, however, can accomplish this and so it may be possible to note and to correlate individual differences or similarities of enzyme behavior which may aid in distinguishing enzyme preparations both as to source, mode of action, and protein or metal content. This is particularly illustrated in part in Fig. 1, where the curves are characteristic of the kind of enzyme preparation under study.

The advantages of the chronometric method

over the manometric method in regard to reliability of results and number of experimental observations necessary have been previously pointed out. This means that the new method can be applied to enzyme studies which were not feasible before because of the time required to obtain a fairly reliable measure of the enzyme.

Summary

1. A chronometric method is described for the measurement of the catecholase activity of tyrosinase using catechol as a substrate in the presence of ascorbic acid.

2. The enzyme activity has been shown to be a function of the time required by the enzyme to produce quinone just in excess of the quantity of ascorbic acid. The presence of quinone is detected by sampling the reaction mixture into a starch–hydriodic acid solution.

3. Curves correlating the chronometric method with the manometric method are given as well as expressions which allow the calculation of the enzyme activity.

4. Results with the chronometric method have been shown to be much more reliable than those obtained using the manometric method and can be obtained with considerably less effort.

5. The behaviors of high catecholase and high cresolase preparations have been compared using the new method. The importance of catechol concentration as an aid in characterizing the enzyme preparations has been pointed out.

6. The behavior of enzyme preparations whose properties resemble in varying degree those of the two main types has been observed using the chronometric method. They have been called intermediate preparations.

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Cleavage of the Alkyl-Oxygen Bond in the Hydrolysis of Esters. *t*-Butyl 2,4,6-Trimethylbenzoate

BY SAUL G. COHEN AND A. SCHNEIDER

It is generally accepted that the hydrolysis of esters of carboxylic acids and the formation of these esters are reactions which involve no rupture of the carbon–oxygen bond of the alcohol.[1] The work of Reid, *et al.*,[1a] on the esterification of thio-acids, the hydrolysis of thio-esters and the esterification of mercaptans gave indirect evidence for this. The sum of information which is available from the hydrolysis of carboxylic esters of optically active alcohols confirms this conclusion. There are many cases in which such esters have been hydrolyzed and there is no simple instance of inversion of configuration of the alcohol.[2] If the alkyl–oxygen bond were involved, it is likely that examples of inversion of configuration would be found. The base-catalyzed hydrolysis of amyl acetate in water enriched in the oxygen isotope of mass 18,[3] and the acid-catalyzed esterification of benzoic acid in methanol enriched in heavy oxygen[4] gave direct evidence that these reactions involve no cleavage of the carbon–oxygen bond of the alcohol.

$$\mathrm{R\overset{O}{\overset{\|}{C}}\!-\!\!\mid\!\!-OR' + HOH \rightleftharpoons R\overset{O}{\overset{\|}{C}}\!-\!\!\mid\!\!-OH + HOR'} \quad (1)$$

These conclusions have been based on a study of the reactions of primary and secondary alcohols and their carboxylic acid esters, and, apparently, are valid generalizations for these classes of compounds. There are a few exceptions in these classes: compounds which because of special structural features, show unusual reactivity. These include β-lactones,[5] the hypothetical α-lactones[6] and an ester of a secondary allylic alcohol.[7]

We have investigated the reactions of some es-

(1) After this manuscript had been prepared, a discussion of this problem appeared in the *Annual Reports* on the Progress of Chemistry for 1940, London, **38**, 1941, 229 ff. This article summarized the evidence which indicated that these reactions involve a rupture of the acyl–oxygen bond, and concluded that little if any contribution to ester hydrolysis is made by cleavage of the alkyl–oxygen bond.

(1a) Reid, *Am. Chem. J.*, **43**, 489 (1910); Pratt and Reid, THIS JOURNAL, **37**, 1934 (1915); Sachs and Reid, *ibid.*, **38**, 2746 (1916).

(2) (a) Fischer, *Ann.*, **394**, 360 (1912); (b) Verkade, *et al.*, *ibid.*, **477**, 287, 297 (1930).

(3) Polanyi and Szabo, *Trans. Faraday Soc.*, **30**, 508 (1934).

(4) Roberts and Urey, THIS JOURNAL, **60**, 2391 (1938); **61**, 2584 (1939).

(5) Olson and Miller, *ibid.*, **60**, 2687 (1938).

(6) (a) Winstein, *ibid.*, **61**, 1635 (1939); (b) Bean, Kenyon and Phillips, *J. Chem. Soc.*, 303 (1936).

(7) (a) Kenyon, Partridge and Phillips, *ibid.*, 85 (1936); (b) Burton and Ingold, *ibid.*, 904 (1928).