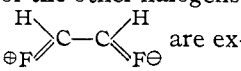
tions were made in the same manner as for the ethanes. Here, as with the ethanes, most of the experimental values are ΔH^0 at around room temperature rather than ΔE_0^0.

It has been recognized that the energy differences between *cis* and *trans* isomers of certain substituted ethylenes are not in accord with the expectation of a repulsion between the substituents.[3] Table II bears out this conclusion strikingly, and shows that even where the expected isomer is the more stable the difference is not as large as expected. A plausible explanation of this fact has been offered by Pitzer and Hollenberg[3] in terms of attraction between formal charges on the chlorine atoms in certain resonance structures which have double bonds between the substituents and the carbon atoms. Table II indicates that this sort of resonance is general for substituted olefins, being of the hyperconjugation type when methyl groups are involved.

By providing a better standard of comparison for the "non-resonating" molecule, the present calculations also permit a better evaluation of the fractional double bond character than was previously possible.[3] From the equations of Pitzer and Hollenberg a 4% double bond character is calculated for the C–Cl bonds in 1,2-dichloroethylene. This estimate is in excellent agreement with recent theoretical[24] and empirical[25] calculations, which yield about 6 and 4%, respectively.

It is noteworthy that the *cis* form of 1,2-difluoroethane cannot be stabilized by the same sort of resonance. Although fluorine can double bond to carbon, it cannot do so by taking up extra electrons because it lacks the d-orbitals of the other halogens, and resonance forms such as  are excluded on energy grounds. It is therefore predicted that the *trans* form of 1,2-difluoroethane will be more stable than the *cis* form by at least several hundred small calories per mole.

Acknowledgments.—The authors wish to thank Mr. Louis Ho for his help with some of the calculations, and Professors R. P. Smith and Henry Eyring for some stimulating conversions.

(24) J. H. Goldstein, *J. Chem. Phys.*, **24**, 106 (1956).

(25) (a) J. H. Goldstein and J. K. Bragg, *Phys. Rev.*, **78**, 347 (1950); (b) J. H. Goldstein and J. K. Bragg, *ibid.*, **75**, 1453 (1949).

MINNEAPOLIS, MINN.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, FLORIDA STATE UNIVERSITY]

A Spectrophotometric Method for the Determination of the Catecholase Activity of Tyrosinase and Some of its Applications[1]

BY MOHAMED ASHRAF EL-BAYOUMI[2] AND EARL FRIEDEN

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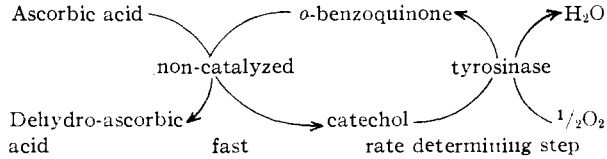
A spectrophotometric method is described for determining the catecholase activity of tyrosinase. Ascorbic acid is used to reduce the *o*-benzoquinone formed from the interaction of catechol with tyrosinase. The enzymatic activity is determined by measuring the decrease in optical density at 265 mμ, the absorption maximum for ascorbate ion. Spectral changes during the reaction are described. Effects of varying substrate and enzyme concentration and *p*H are reported. Applications of the method to tyrosinase inhibition and proteolysis studies are given. The use of this method and an analogous method employing ascorbic acid oxidase for ascorbic acid analysis in biological materials such as orange juice is also described.

Introduction

Available methods for the study of the catalytic activity of the enzyme tyrosinase leave much to be desired.[3] Conventional Warburg technique using catechol or other phenols as substrate results in the formation of highly reactive products, which lead to a highly complex reaction mechanism.[3] While the determination of initial rates would obviate this difficulty, the Warburg method is poorly adapted for such rate studies. A satisfactory method is the "chronometric" method of Miller and Dawson[4] which employs ascorbic acid as an inert reductant[5a] to reduce continuously and instantaneously the quinone back to catechol.



Net reaction: Ascorbic acid + $\frac{1}{2}O_2 \rightleftharpoons$ Dehydroascorbic acid + H_2O

The maintenance of the catechol in the reduced state effectively eliminates the possibility of complicating secondary reactions involving the quinone. However, the technique used to follow the disappearance of the ascorbate was cumbersome. In addition, this method is not easily adaptable to numerous kinetic studies of tyrosinase because of the complexity of the rate equations.[4] We have, therefore, adapted this method to a highly sensitive and rapid determination of the catecholase ac-

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(2) The major portion of this work is from the thesis of M. Ashraf El-Bayoumi for the M.S. degree in chemistry, Florida State University, January, 1957.

(3) J. M. Nelson and C. R. Dawson, *Advances in Enzym.*, Vol. 1, 4, 99 (1946); C. R. Dawson and W. B. Tarpley in *The Enzymes*, **II**, 1, 464 (1951).

(4) W. H. Miller and C. R. Dawson, THIS JOURNAL, **63**, 3368 (1941); L. Ingraham and B. Makower, *Anal. Chem.*, **27**, 916 (1955).

(5) (a) L. Ingraham, THIS JOURNAL, **78**, 5095 (1956); (b) L. Ingraham, *Anal. Chem.*, **28**, 1177 (1956).

tivity of tyrosinase using a spectrophotometric measurement to follow the disappearance of ascorbate. Recently, Ingraham[6b] used a polarized rotating platinum electrode to determine polyphenol oxidase activity at various ascorbate concentrations. Conveniently, the oxygen consumption was recorded potentiometrically.

Results

Effect of Tyrosinase Concentration.—The catecholase activity at different enzyme concentrations is shown in Figs. 1 and 2. The optical density of

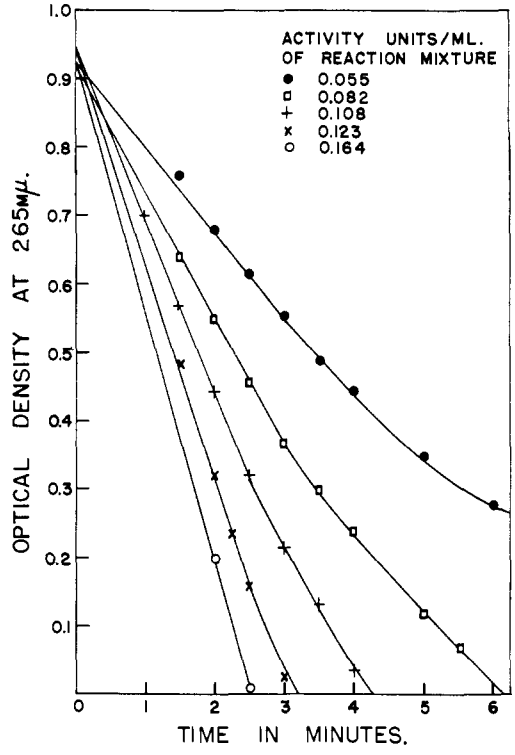

Fig. 1.—The catecholase activity of tyrosinase at different enzyme concentrations. The reaction mixture contained in each case 7.0×10^{-5} M ascorbate, 2.0×10^{-6} M EDTA, 1.66×10^{-4} M catechol at pH 7.2.

ascorbate is a linear function of its concentration at its absorption maximum of 265 mμ. The rate of change of the optical density may be taken as a measure of the catecholase activity of tyrosinase since for every one-half mole of oxygen which disappears, one mole of ascorbate also disappears. More details of the spectral changes occurring during the reaction are presented in the Experimental section. The precise determination of the rate for each enzyme concentration may be accurately made by obtaining the change in optical density per unit time for the linear portion of the curve (usually 1–3 minutes). Under some conditions a first-order rate constant with respect to ascorbate disappearance must be calculated. It should be noted that in Fig. 1, the catechol concentration employed was 1.61×10^{-4} M which is not a saturating substrate concentration.

The rates obtained from Fig. 1 show the expected linearity between the enzyme activity and enzyme concentration. This verifies the usefulness of the

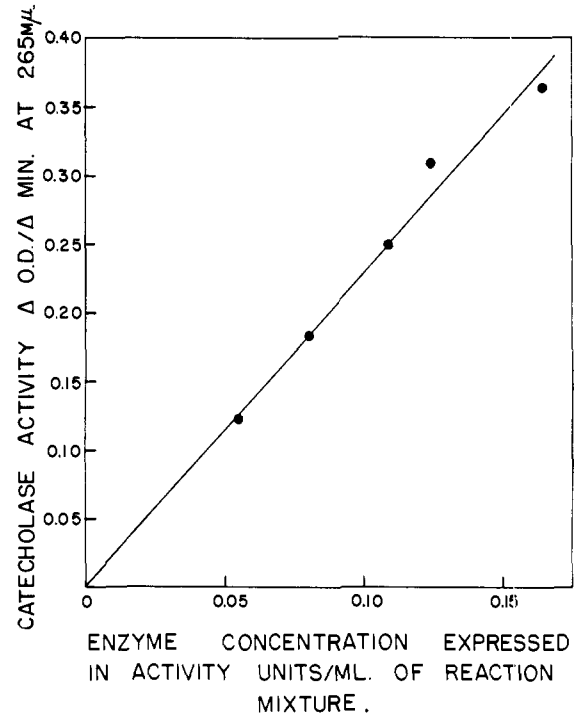

Fig. 2.—Effect of enzyme concentration on the catecholase activity of tyrosinase. Conditions are identical with those in Fig. 1. The activity unit previously has been defined as the amount of enzyme capable of catalyzing oxygen uptake at a rate of 10 μl. per minute at 37°.

method in establishing tyrosinase concentration by measuring its catecholase activity. It should be emphasized that with the catechol concentration used the enzyme is operating only at about one-half its maximum velocity.

Effect of Catechol Concentration.—The effect of catechol concentration is shown in Fig. 3. The substrate curve conforms with Michaelis–Menten kinetics as indicated in Fig. 3 and Lineweaver–Burk

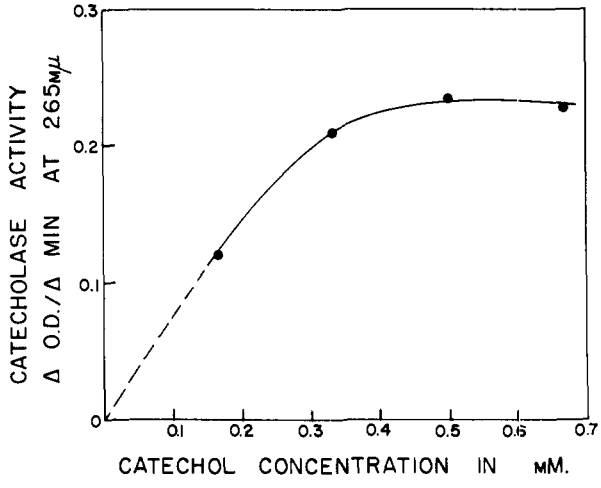

Fig. 3.—Effect of catechol concentration on the catecholase activity of tyrosinase. Final enzyme concentration used was 0.055 unit per ml. The reaction mixture contained 7.0×10^{-5} M ascorbate, 2.0×10^{-6} M EDTA at pH 7.2.

plots. The calculated K_m for catechol at 25° is 1.7×10^{-4} M, a value lower than any previous value reported. A K_m value of 5×10^{-4} M for catechol was estimated from the data of Ingraham using a titrimetric method.[5] Warner,[6a] using the chronometric method,[4] reported a K_m of 2.8×10^{-4} M. The above values are for the mushroom enzyme. For the potato enzyme, however, values of $4.6\text{–}5.0 \times 10^{-3}$ M have been reported.[6a,6b] Warner attributed these differences to either inherent differences in the two enzymes or to the presence of a competitive inhibitor in the potato enzyme. Earlier Miller, *et al.*,[6c] noted that the K_m is a function of the purity of polyphenol oxidase. It is of interest to note that frequently spectrophotometric methods give lower K_m values, as previously noted for another copper enzyme, ascorbic acid oxidase.[6d]

In as much as no inhibiting catechol oxidation products are present, it is possible and frequently convenient to employ more saturating catechol concentrations such as 5×10^{-4} M. No effect was observed for the ascorbate on the rate of the reaction in agreement with Ingraham.[5a] An ascorbate concentration convenient for optical density readings was selected.

Effect of *p*H on Tyrosinase.—An optimum in the *p*H 7.0 region was found using phosphate buffer. These results are similar to those observed by Miller and Dawson[4] who reported an optimum between *p*H 5.5 and 7.0. However, relatively small decreases in enzyme activity are observed at adjacent *p*H's. Thus the activity at *p*H 6 and *p*H 8 was 90 and 97% of the activity at *p*H 7.0, respectively. Similar activity–*p*H curves have been noted for tyrosinase and the related copper enzyme, ascorbic acid oxidase.[7]

Stability of the Catecholase Activity of Tyrosinase.—The catecholase activity of tyrosinase showed unexpected stability when its activity was studied by this spectrophotometric method. Figure 4 shows the effect of storage at 30° of solutions of highly purified tyrosinase at various *p*H's. Over 90% of the activity was retained over 25 hours at *p*H 6.95. Loss of activity was more pronounced at other *p*H's, the order of stability of the enzyme at all *p*H's tested being $6.95 > 5.95 > 7.95 > 5.33$. Since the enzyme preparation was estimated as 50% pure, the stability of tyrosinase found in these studies cannot be attributed to the presence of extraneous proteins or to the self protection sometimes afforded by high protein concentration.

Some Applications of the Method

Inhibition Studies.—The spectrophotometric method for tyrosinase activity has been found to be suitable for an extensive study of the effect of certain inhibiting reagents on its catecholase activity. The effect of specific cuprous and cupric reagents on tyrosinase using this method has recently been reported.[8] For the classical iron and copper enzyme inhibitor, cyanide, it was found that approximately 6×10^{-5} M cyanide ion is required for 50% inhibition of the catecholase activity of tyrosinase. Using the Warburg technique, Du Bois and Erway[9] reported 50% inhibition with about 1×10^{-4} M cyanide. Further experiments on the nature of cyanide ion inhibition show that, as expected, the inhibition appears to be noncompetitive. It is possible that even these low concentrations of cyanide removes the Cu ion from the enzyme protein, since cyanide ion shows evidence of irreversible inhibition as indicated by the Ackermann–Potter method.[10] Kubowitz[11] reported long ago that cyanide removed Cu ion from potato polyphenol oxidase.

Proteolysis of Tyrosinase.—In order to study and isolate the active site of tyrosinase, the spectrophotometric method was used to follow the proteolysis of tyrosinase by proteolytic enzymes. The extent of hydrolysis was measured by trichloroacetic acid (TCA) soluble nitrogen and the remaining tyrosinase activity determined as a function of the time of incubation at 30°. The appearance of TCA soluble peptides was most significant with 30 mg. of chymotrypsin at *p*H 7.95 and was paralleled by the loss in catecholase activity. Ethylenediaminetetraacetate (EDTA) (0.4 M) reduced the loss of activity, perhaps due to the chelation of the Cu ion at the active site of tyrosinase. An activating effect of chymotrypsin was noted at *p*H 5.95 and might be due to an unmasking effect of the protease at this *p*H or perhaps to a non-specific protein effect previously noted for tyrosinase.[4]

Ascorbic Acid Analysis using Tyrosinase and Ascorbic Acid Oxidase.[12]—Considerable attention has been paid to the analysis of ascorbic acid. The principal methods used involve the titration of the dienol with a reducible dye such as 2,6-dichlorophenolindophenol and the formation of an osazone with 2,4-dinitrophenylhydrazone. These chemical methods are summarized in several recent laboratory handbooks.[13] They suffer from the lack of specificity of the reactions employed since substances other than ascorbate undergo similar reactions. The possibility of a more specific enzymatic method for ascorbate analysis was suggested some years ago by Tauber.[14]

Under the proper conditions the spectrophotometric method for tyrosinase can be modified to serve as a sensitive method for the determination of ascorbic acid. The method affords a sensitivity equal to if not greater than the common

(6) (a) C. Warner, *Australian J. Sci.*, **B4**, 554 (1951); (b) H. Heymann, Z. Rogach and R. L. Mayer, THIS JOURNAL, **76**, 6330 (1954); (c) W. H. Miller, M. F. Mallette, L. J. Roth and C. R. Dawson, *ibid.*, **66**, 514 (1944); (d) E. Frieden and I. Maggiolo, *Biochem. Biophys.*, **48**, 448 (1957).

(7) "Copper Metabolism," edited by W. E. McElroy and B. Glass, The Johns Hopkins Press, Baltimore, Md., 1950.

(8) E. Frieden, G. Ezell and Y. Karkhanis, *Federation Proc.*, **16**, 183 (1957). A more detailed report is in preparation.

(9) K. P. Du Bois and W. F. Erway, *J. Biol. Chem.*, **165**, 711 (1946).

(10) W. W. Ackermann and V. R. Potter, *Proc. Soc. Exptl. Biol. Med.*, **72**, 1 (1949).

(11) F. Kubowitz, *Biochem. Z.*, **299**, 32 (1939).

(12) We are indebted to Mrs. Jane Crosby for much of the experimental work described in this section.

(13) J. H. Rose, in "Methods of Biochemical Analysis," edited by D. Glick, Interscience Publishers, New York, N. Y., 1954, pp. 115–150; "Methods of Vitamin Assay," published by The Association of Vitamin Chemists, 1947, pp. 143–169.

(14) H. Tauber, I. S. Kleiner and D. Mishkind, *J. Biol. Chem.*, **110**, 211 (1935).

chemical methods employed for ascorbic acid analysis.

Simultaneously, in a study of some of the properties of ascorbic acid oxidase (AAO),[15] we have had occasion to extend the spectrophotometric method for determining AAO activity suggested in brief by Racker.[16] Conditions also were noted which were suitable for an additional enzymatic method for ascorbic acid.

Both enzymatic methods were compared with the dye-titration method for the determination of the ascorbic acid in frozen orange concentrate. As shown in Table I, slightly lower but comparable results were obtained with the two enzymatic methods. The higher value for the dye method may indicate a lack of specificity for the common chemical methods employed for ascorbate assay. Accordingly the use of the suggested enzymatic methods may lead to the development of a more specific, sensitive and rapid measurement of ascorbic acid analysis in orange concentrate and other biological materials such as blood and tissue. It is anticipated that the methods could be appropriately modified to permit the concomitant determination of dehydroascorbate. Of all the possible methods mentioned, the ascorbic acid oxidase should be the most specific because of the narrowness of the substrate spectrum of this enzyme.

TABLE I

COMPARATIVE ASCORBIC ACID ANALYSIS OF FROZEN ORANGE CONCENTRATE BY ENZYMATIC METHODS

Method	Ascorbic acid, mg./g. orange concentrate
Dye-titration	1.78 ± 0.04
Tyrosinase	1.69 ± .02
Ascorbic acid oxidase	1.70 ± .03

Experimental

Catecholase Activity Determination.—The catecholase activity of tyrosinase was determined at 25° (except where noted) at the indicated *p*H by determining the rate of decrease in optical density of ascorbate at 265 mμ in a Beckman DU spectrophotometer. Typical composition of the reaction mixture and reference cells are given in Table II. It should be noted that these compositions were used in the activity determination of tyrosinase in various studies except in the study of the effect of catechol and enzyme concentration on rate. In the latter case the final concentration of the various constituents of the reaction mixture are indicated in the corresponding figures (Fig. 1, 3). A stopwatch was started at the same time as the enzyme was stirred into the reaction mixture. No precautions were taken to keep air away from the solution. Readings were taken at suitable intervals and the rate determined from appropriate plots similar to those shown in Fig. 1. Under other conditions, it has been found necessary to treat the data as representing a first-order reaction with respect to ascorbate. Accordingly, for linearity here, a plot of the log of the optical density *versus* the time may be used.

Optical Density Changes during the Reaction.—The spectrum of the reaction mixtures used to determine catecholase activity was determined before adding the enzyme and at several intervals of time after its addition (Fig. 5) using a recording Beckman DK Spectrophotometer. The reference cell contained all components present in the reaction cell except ascorbate and enzyme. The spectra of the same concentration of ascorbate and catechol solutions taken at the same *p*H are also shown in Fig. 5 (curves 1 and 2). Curve 3 shows the spectrum of the reaction mixture

(15) E. Frieden and I. Maggiolo, *Biochem. Biophys. Acta*, in press (1957).

(16) E. Racker, *ibid.*, **9**, 577 (1952).

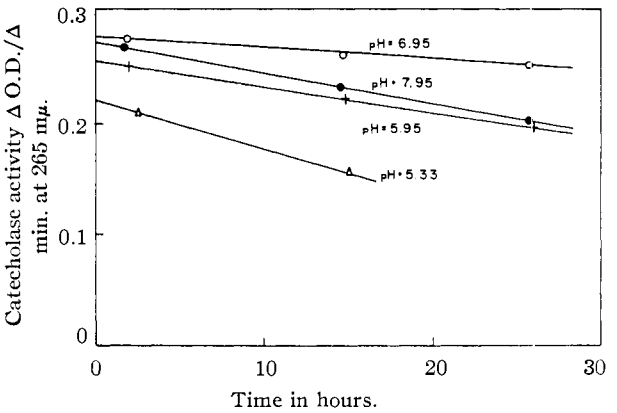

Fig. 4.—Stability of catecholase activity at 30° at different *p*H values. Enzyme solutions were stored at an identical concentration of 240 units/ml.

before the enzyme was added. Curve 4, Fig. 5, records the spectrum obtained 20 sec. after tyrosinase was added. A marked reduction in ascorbate absorption is already noticeable. The peak has shifted from 265 to 278 mμ due to the contribution of the catechol absorption at 278 mμ. Curve 5 was taken after the reaction was complete and shows the complete disappearance of the ascorbate peak at 265 mμ.

TABLE II

COMPOSITION OF REACTION MIXTURES FOR MEASURING THE CATECHOLASE ACTIVITY OF TYROSINASE

Reagent	Concn., *M*	Vol. of the components of the reaction cuvette	Vol. of the components of the ref. cuvette
Ascorbic acid	2.14×10^{-4}	0.10	..
E.D.T.A.[a]	5.2×10^{-5}	.9	0.9
Catechol	1.00×10^{-3}	.1	.1
Phosphate buffer[b]	0.067	.2	.2
Tyrosinase[c] in redistilled water	12 units/ml.	.10	..
Redistilled water		1.6	1.8
Total volume		3.0	3.0

[a] Ethylenediaminetetraacetic acid (EDTA), generously provided by the Bersworth Chemical Company, was used to prevent the autoöxidation of ascorbic acid. [b] Phosphate buffers of various *p*H's were used as indicated. [c] Tyrosinase used in most of these experiments was obtained from the Reheis Company, Berkeley Heights, New Jersey. Each preparation contained over 0.2% copper and 1500 units/mg. corresponding to a preparation well over 50% pure and approaching 90% purity. A unit of tyrosinase activity is defined as the amount of enzyme capable of catalyzing oxygen uptake at a rate of 10 u.l. per minute as defined by Mallette and Dawson.[17]

In fact, it was noted that the O.D. of the ascorbate measured directly at 265 mμ is equal to the O.D. change at 265 mμ as determined in a complete rate measurement. This includes a correction for a change in O.D. due to the oxidation of the catechol. This agreement justifies the application of the method to the determination of ascorbic acid in natural products containing no substance other than ascorbic acid that can reduce *o*-benzoquinone. Apparent anomalies in the various curves in the region around and below 250 mμ are believed due to some interactions between EDTA and various other components such as catechol. This type of interaction was shown in independent experiments not included in Fig. 5.

Stability Measurements.—In determining the stability of tyrosinase a solution containing 240 units per ml. was incubated at 30° at the indicated *p*H. Aliquots were with-

(17) M. F. Mallette and C. R. Dawson, THIS JOURNAL, **69**, 466 (1947).

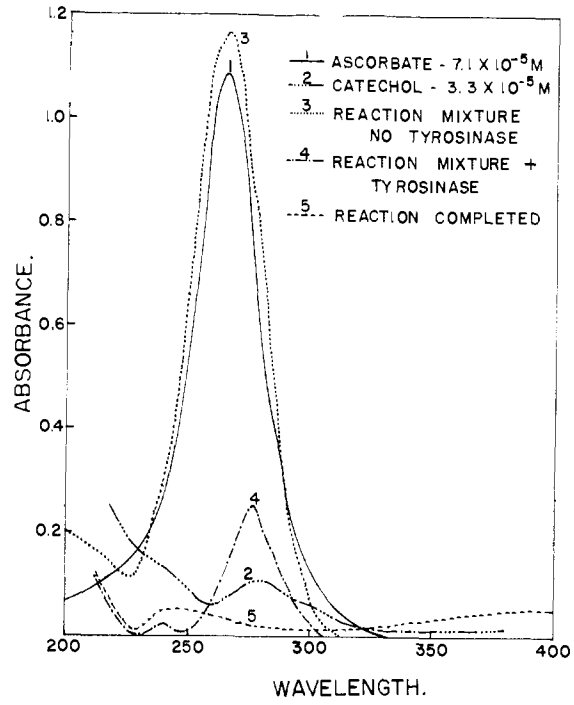

Fig. 5.—Ultraviolet absorption of ascorbate, catechol and the catecholase reaction mixture at various times. The reference cell contained corresponding concentrations of EDTA and 0.067 *M* phosphate buffer, *p*H 6.7.

drawn at appropriate time intervals for tyrosinase measurements.

Inhibition Studies.—To study tyrosinase inhibition, the inhibitor is added to the reaction mixture prior to the addition of the enzyme. Dilutions of a standard 0.10 *M* sodium cyanide solution were prepared fresh for use within several days and kept in a tightly stoppered container. For these particular experiments a crude tyrosinase of approximately 10 units/mg., purchased from the Worthington Biochemical Co., Freehold, N. J., was used.

Proteolysis of Tyrosinase.—Crystalline chymotrypsin, purchased from the Worthington Biochemical Co., Freehold, New Jersey, was used. The indicated amounts of chymotrypsin were added to tyrosinase solutions and the *p*H adjusted with phosphate buffer to the final indicated *p*H. Aliquots were withdrawn at the appropriate time for tyrosinase activity and trichloroacetic acid soluble nitrogen. The TCA soluble nitrogen was determined by digestion and direct nesslerization of the supernatant obtained by centrifuging an equal volume of 10% TCA with an equal volume of digestion mixture.

Ascorbic Acid Analysis

Dye-titration Method.—The ascorbate in orange concentrates was titrated with the dye, 2,6-dichlorophenolindophenol, by the routine method as described by the Association of Vitamin Chemistry.[13] When pure samples of ascorbic acid were used to standardize the dye, the vitamin was dissolved in 3.0×10^{-4} *M* EDTA and 3% phosphoric acid.

Enzymatic Methods. Preparation of the Samples.—An approximate 2-gram sample of frozen orange juice concentrate containing approximately 3 to 4 mg. of ascorbic acid is weighed to the desired accuracy into a 100-ml. volumetric flask. About 50 ml. of EDTA (6.0×10^{-4} *M*) is added and the 100-ml. volume completed with 6% metaphosphoric acid. The solution is centrifuged for 10 min. The supernatant may be kept safely at air conditioned room temperature for several hours. Usually 1.00 ml. of this supernatant is diluted with a solution containing 3.0×10^{-4} *M* EDTA and 3% metaphosphoric acid and the *p*H adjusted to 6.0 with $NaHCO_3$ powder and a final volume of 10.0 ml. attained. The volume of this sample can be adjusted depending upon the subsequent optical density (O.D.) reading. The final concentration of ascorbic acid should be in the range of $1\text{–}3 \times 10^{-5}$ *M*. 3.00 ml. of the diluted supernatant is then transferred to a 1-cm. Beckman cell.

Tyrosinase Method.—To the 3.00-ml. sample, 0.1 ml. of 5.0×10^{-3} *M* catechol is added. The O.D. is read and 5 u.l. of tyrosinase (2000 units/ml.) is added. The O.D. is then determined after no further change has occurred which is always less than five minutes. After subtraction of a catechol–tyrosinase blank, the amount of ascorbate may be calculated from the O.D. increment and appropriate dilution factors.

Ascorbic Acid Oxidase (AAO) Method.—5–6 units (Lovett-Janison) AAO (Reheis Chem. Co.) are added with a micro pipet, usually comprising 10 λ so that no significant dilution occurs. If significant dilution is necessary, an appropriate correction factor can be applied. The conversion of ascorbic acid to dehydroascorbic acid occurs rapidly and is complete within five minutes. The final O.D. is read and the decrease in O.D. can be converted to ascorbic acid from an O.D. ascorbate standard curve or from the molar extinction coefficient.

Addendum.—After this article was submitted for publication, it has come to our attention that Drs. K. Yasonobu and W. B. Dandliker of the Department of Biochemistry, University of Washington, School of Medicine, Seattle, have also developed a similar spectrophotometric method for determining polyphenol oxidase activity.

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