this problem, invertase was added to a cellulose column and washed with the *aqueous* phase only. All the invertase quickly passed through the column. Enzyme adsorption is *not* playing an important role in these tests.

Since the enzyme is not adsorbed, the role of the solid phase in the column must be mechanical; to hold the aqueous phase in position and to maintain a maximum interfacial surface. To demonstrate this[8] the cellulose was eliminated by using a U-tube with a coarse sintered-glass filter at the base of one arm. This arm was partly filled with small glass beads and 4 ml. of invertase in the aqueous phase was placed in it. To the other arm was added sucrose in 1-butanol. A head of solvent was maintained so that 15–60 ml. of solvent per hour passed through the filter and rose through the aqueous phase as fine droplets, then overflowed into a collector. Under these conditions the sucrose (2 mg./ml. of solvent) was hydrolyzed completely with the formation of the same products as were produced on the column.

To test whether enzymes remain stable under such drastic conditions, an invertase column was set up. Sucrose, 3 mg. per ml. in 1-butanol, was passed through the column continuously for 28 days. The flow rate averaged 11 ml. per hour. Aliquots were collected daily and the per cent. of hydrolysis of the sucrose was determined.

While there was considerable day to day variation, probably due to fluctuations in the flow rate and in room temperature, there was no significant decrease in rate of hydrolysis over this period.

TABLE I

HYDROLYSIS OF SUCROSE BY INVERTASE

Day	% Hydrolysis	Day	% Hydrolysis	Day	% Hydrolysis
1	86	10	53	21	45
2	69	11	54	22	48
3	59	14	76	23	74
4	63	16	70	24	92
7	65	17	65	25	76
8	62	18	62	28	87
9	67				

(8) This modification was suggested by our colleague, Dr. J. D. Loconti.

Discussion

Enzymes are capable of acting continuously in a column operating on the principles of partition chromatography. We believe that the method is applicable to a wide range of enzymes and substrates and perhaps even to catalysts other than enzymes. The necessary details must be worked out for each system. One major problem is the selection of a solvent. Investigations on counter current distribution may suggest suitable solvents. Substrate and products should be reasonably soluble in both phases. The solvent used must not react with either substrate or products, except in certain types of transfer reactions. The enzyme must be reasonably stable under the conditions which exist on the column.

The fact that water quickly elutes the enzyme from the column shows that the enzyme is not adsorbed but exists free in the aqueous phase. As a result, the procedure is not limited to the use of enzymes in columns but may be used in any system involving a partitioning effect, such as the Craig countercurrent distribution apparatus, or any system involving continuous solvent extraction of an aqueous phase.

NATICK, MASS.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

The Effect of Ascorbic Acid on the Inactivation of Tyrosinase[1]

BY WALTER SCHARF AND CHARLES R. DAWSON

RECEIVED MARCH 29, 1958

Reports that the inactivation of the enzyme tyrosinase, during the aerobic oxidation of catechol, is reversed by ascorbic acid have been investigated and found to be illusory. It has been found that deceptive results may arise during colorimetric measurements of the reaction rate from (a) neglecting to keep the reaction-mixture continuously supplied with oxygen and (b) overlooking the fact that the initial oxidation product, *o*-benzoquinone, whose absorbance at 390 mμ forms the basis of the colorimetric method, is very unstable in aqueous solution. Experiments conducted under adequate conditions of oxygen supply lead to the conclusions that (a) inactivation of tyrosinase in the reaction-mixture is not caused by a product reducible by ascorbic acid, and so is not reversible by the latter, and (b) ascorbic acid, in the concentrations used for activity measurements by the chronometric method, has no activating effect on the enzyme.

The copper-enzyme, tyrosinase (polyphenol oxidase), of the mushroom suffers early and progressive inactivation during its catalytic participation in the oxidation of catechol to *o*-benzoquinone by molecular oxygen. The phenomenon has been the subject of much speculation and research,[2–5] as it is distinct in character from the more general type of enzyme inactivation due to environmental factors. As the result of experiments in these laboratories several years ago, Ludwig and Nelson,[2] and Miller, *et al.*,[6] reported that ascorbic acid has no effect on the activity of the enzyme. Consequently the acid has been widely used as a reducing agent in the reaction-mixture during catecholase activity measurements by the chronometric methods.[6,7] This innocuous role for ascorbic acid

(1) From a dissertation submitted by Walter Scharf in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University, 1957.

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

(3) L. J. Roth, Dissertation, Columbia University, 1944.

(4) I. Asimov and C. R. Dawson, THIS JOURNAL, **72**, 820 (1950).

(5) L. L. Ingraham, J. Corse and B. Makower, *ibid.*, **74**, 2623 (1952).

(6) W. H. Miller, M. J. Mallette, L. J. Roth and C. R. Dawson, *ibid.*, **66**, 514 (1944).

(7) W. H. Miller and C. R. Dawson, *ibid.*, **63**, 3375 (1941).

has been questioned recently by Ponting,[8] who reported that the vitamin, when added to a solution in which tyrosinase has become inactivated, is able to reactivate the enzyme. Because of the important implications of Ponting's conclusion, particularly in regard to the validity of the chronometric method, it has seemed advisable to re-evaluate the effect of ascorbic acid on the inactivation of tyrosinase.

Since *o*-benzoquinone, the initial oxidation product of catechol, is unstable in the reaction-mixture,[9] workers who measure the rate of the enzymatic oxidation of catechol by following the quinone absorbance at 390 mμ can be led to erroneous conclusions regarding the activity of the enzyme. Also, when no provisions are made for aerating the solution during such colorimetric rate measurements, the enzymatic reaction may be prematurely slowed down due to exhaustion of the dissolved oxygen supply. As a result of either or both of these factors, the development of a zero rate of quinone formation, as detected colorimetrically during the enzymatic oxidation of catechol, cannot be used as a criterion of complete enzyme inactivation. In this respect, Ponting's experiments and his conclusions are subject to serious criticism. He did not prove that the enzyme had been completely inactivated at the time he added the ascorbic acid, and consequently the restoration of quinone production following the ascorbic acid addition undoubtedly was due to active enzyme in the system at the time he added the reductant.

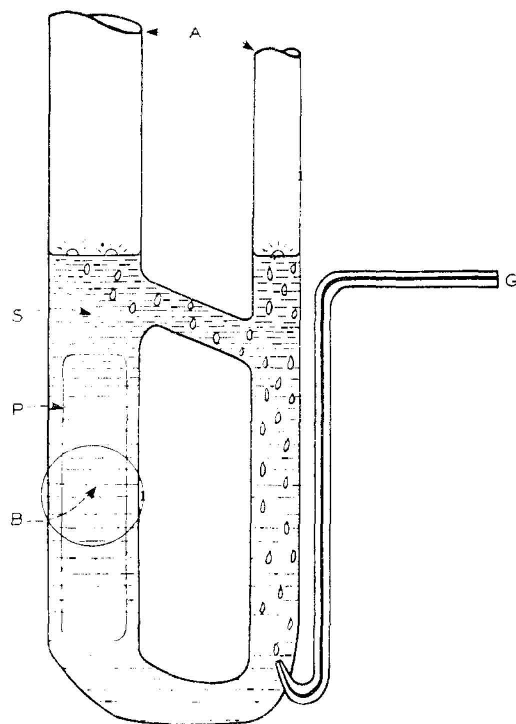

Fig. 1.—Special spectrophotometer-reaction cell providing for continuous aeration during the enzymatic oxidation of catechol: A, gas escape vents and/or liquid addition tubes; B, position of incident beam or monochromatic light perpendicular to plane of paper; G, 1 mm. capillary gas inlet tube; P, optically parallel glass windows providing a 1 cm. light path through enzyme–substrate solution S.

(8) J. D. Ponting, THIS JOURNAL, **76**, 662 (1954).

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

The experiments described in this communication have been designed to prove the above contention, *i.e.*, that Ponting's reactivation effects were artifacts of his special method of following the enzyme activity. By use of a special absorption cell, designed to permit continuous oxygenation of the solution while spectrophotometric measurements were in progress (Fig. 1), it has been found that ascorbic acid cannot reactivate truly spent enzyme or activate functioning enzyme under the conditions employed for activity measurements by the chronometric method.

Experimental

Enzyme.—The tyrosinase used in this investigation was high catecholase preparation No. C400, having a specific activity of 1690 units per milligram and 1170 units per microgram copper. The enzyme was isolated from the common mushroom, *Psaliota campestris*, by the procedure of Mallette, *et al.*[10] Catecholase activity was measured by the chronometric method of Miller, *et al.*,[6] and copper content by a new method recently developed in these laboratories.[11] Appropriate dilutions of the master enzyme solution (containing 15,900 units per milliliter) were made up in ice-cold, redistilled water, just prior to use, and stored at 0–5° for not more than 0.5 hr.

Catechol.—The product (m.p. 104–105°) of the Eastman Kodak Company was found to give a sufficiently colorless aqueous solution to be used in spectrophotometric measurements without further purification.

L-Ascorbic Acid.—Ascorbic acid, obtained from Charles Pfizer and Company (m.p. 189–190°), was used without further purification. Aqueous solutions of this compound were always made up with 1% disodium ethylenediaminetetraacetic acid (Sequestrene Na2, Geigy Co., New York)[12] to minimize autoxidation induced by heavy metal ions.

Ceric Sulfate.—Ceric sulfate solutions were prepared by dissolving analytical grade ceric ammonium sulfate [$Ce(SO_4)_2 \cdot 2(NH_4)_2SO_4 \cdot 4H_2O$; Fisher] in water made 0.2 *M* with sulfuric acid, to prevent hydrolysis. When addition of aliquots of these solutions to buffered systems caused acidification in excess of 0.02 *p*H unit, the *p*H was readjusted to the desired value with 0.4 *N* sodium hydroxide.

Copper-free Water.—All solutions were made up in distilled water which had been redistilled through an all Pyrex glass still, equipped with a hot-wire film breaker to prevent contamination of the distillate by entrainment. Such distilled water has been shown to contain less than 0.01 microgram of Cu^{++} per ml.[13]

The Spectrophotometric Measuring Procedure.—The absorbance *vs.* time curves shown in the figures were obtained by use of a Cary Recording Spectrophotometer (Model 11, Series 62, Applied Physics Corp., Pasadena, Calif.). In addition to standard 1 cm. silica spectrophotometer cells, a special absorption cell (Fig. 1), which permitted continuous bubbling of air through the solution while optical measurements were in progress, was employed in certain experiments. The rate of air flow was maintained at *ca.* 10 liters/hr. The ambient temperature could not be controlled but was never found to vary more than 0.5° during the course of a run. The curves shown in the figures were obtained by replotting, on reduced scales, the pen tracings from the recorder chart.

Results and Discussion

Figure 2 shows the reactivation of color formation that occurred (following a preliminary bleaching) when ascorbic acid was added to the enzyme–cate-

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

(11) G. Stark and C. R. Dawson, *Anal. Chem.*, in press.

(12) That this substance has no other effect on the enzyme or ascorbic acid was shown by the fact that the same chronometric endpoint time was obtained whether Na2EDTA was present in the reaction-mixture or not.

(13) R. J. Magee, Dissertation, Columbia University, 1954.

chol reaction mixture, after the rate of development of quinone color had decreased to nearly zero (curve 1). This resurgence of quinone color, as followed spectrophotometrically at 390 mμ, was ascribed by Ponting[8] to enzyme reactivation by ascorbic acid. However, it is apparent from curve 2, Fig. 2, that simply supplying more oxygen to the

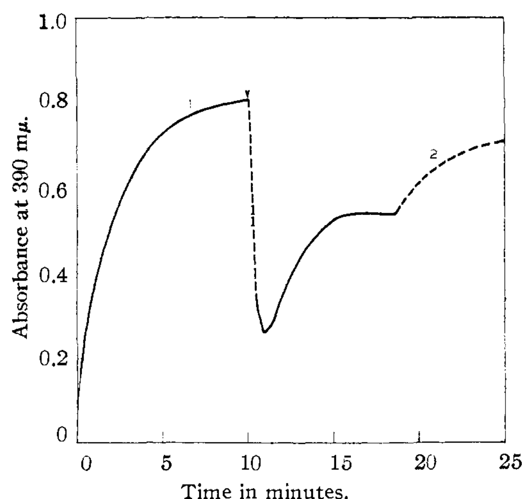

Fig. 2.—Showing the enzymatic oxidation of catechol to *o*-benzoquinone as followed spectrophotometrically before and after adding ascorbic acid. Curve 1: a 3-ml. sample of 0.1 *M* catechol in 0.1 *M* acetate buffer, *p*H 5.1 was added at zero time to 0.1 ml. of enzyme solution (4.5 catecholase units) in a 1 cm. spectrophotometer cell. At the ten minute point of 0.06 ml. sample of ascorbic acid solution containing 1.0 mg. of ascorbic acid per ml. was introduced and the contents of the cell were quickly mixed by capping and tipping three times. Note that color formation resumed after a preliminary bleaching. Curve 2: at the 18 minute point, the cell was again removed from the instrument, capped, tipped three times and replaced. Note that the O.D. rises again following the mixing with air; temperature 27.1 ± 0.5°.

spectrophotometer cell (by mixing; no ascorbic acid added) caused a redevelopment of quinone color after the rate had approached zero. In other words, the lack of quinone production between the 15 and 18-minute period was due to an inadequate supply of oxygen rather than due to inactivation of the enzyme. Because of the small air–liquid interface available and the absence of agitation in spectrophotometric measurements using ordinary 1 cm. cells, any rapid oxygen-consuming reaction will quickly exhaust the available supply of this gas in the solution and so come to a premature halt. The addition of ascorbic acid at the 10-minute point also resulted in the addition of oxygen to the system. The renewed *o*-benzoquinone production which followed cannot therefore be ascribed to reactivation of dead enzyme by ascorbic acid.

Ponting reported that "calculations showed that —oxygen was not a limiting factor" in his experiments. Since he probably based his calculations on the *apparent* amount of quinone produced, it is not surprising that his estimate of the available excess of oxygen in his system was low. Due to the instability of *o*-benzoquinone in aqueous solution,[9,14] the spectrophotometer readings taken during the oxidation reaction represent only a fraction of the quinone actually produced in the system at any given time. Furthermore, this fraction decreases as the time of the reaction increases. This point will be elaborated in the discussion of the following experiments.

The data represented in Fig. 3 were obtained under conditions of ample oxygen supply using the special bubbler spectrophotometer-reaction cell diagrammed in Fig. 1. In curve 1, where the ascorbic acid was added relatively early in the reaction while the quinone production was still weakly apparent, an initial bleaching was followed by a resurgence of color. In curve 2, however, the ascorbic acid was not added until much later when the quinone color was actually falling off. By this time the enzyme was completely inactivated, and the bleaching effect of the ascorbic acid was not followed by renewed color development. It is apparent that ascorbic acid did not reactivate truly spent enzyme.

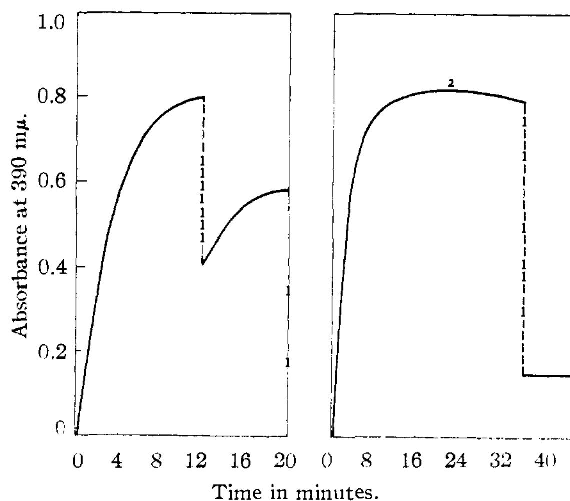

Fig. 3.—Showing the difference in effect of ascorbic acid on the quinone color developed at an early and a late stage in the enzymatic oxidation of catechol under conditions of ample oxygen supply. Curve 1: a 10-ml. sample of 0.1 *M* catechol in 0.1 *M* acetate buffer, *p*H 5.1 was added at zero time to 0.1 ml. of enzyme solution (4.0 catecholase units) in the special spectrophotometer cell (Fig. 1). At zero time the air supply was turned on, and at the 12 minute period a 0.1 ml. sample of ascorbic acid solution containing 1.0 mg. of ascorbic acid per ml. was added. Curve 2: same as curve 1 except that the ascorbic acid was added after 35 minutes when the quinone O.D. curve was falling. Note that no redevelopment of color followed the bleaching effect of the ascorbic acid as it did in the case of curve 1; temperature 26.7 ± 0.5°.

The experiments of Fig. 3 focus attention on the fact that *o*-benzoquinone is unstable in aqueous solution. Thus the course of the reaction as followed spectrophotometrically is merely the result of two competing reactions, *i.e.*, the enzymatic production of quinone and the loss of quinone *via* reaction with water.[14] As long as the quinone–time curve shows a positive slope, the enzyme is active and producing quinone faster than it disappears.

(14) W. B. Tarpley, Dissertation, Columbia University, 1951.

When the slope becomes zero and runs parallel to the time axis, the enzyme must still be active and producing quinone at a rate equal to its disappearance. In other words, a steady state may be maintained for a considerable period of time if the amount and stability of the enzyme is such that its inactivation only slowly becomes apparent. This undoubtedly explains why Ponting found a rapid renewal of color development even after five bleachings with ascorbic acid over a period of an hour. He was using a relatively large amount of a partially purified apple enzyme which apparently was not inactivated rapidly during the reaction. It follows from the above that only when the slope of the quinone–time curve becomes negative can it be judged that the enzyme has been significantly inactivated.

Other factors that markedly influence the stability of *o*-benzoquinone in aqueous solutions containing catechol are the concentration of quinone, the concentration of catechol and the *p*H.[9] The effect of quinone concentration is shown in Fig. 4. It

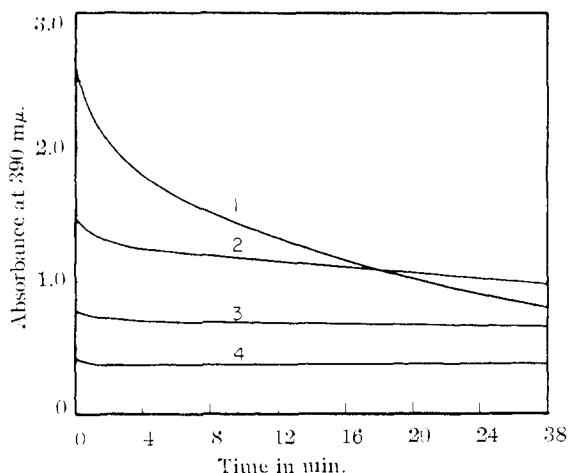

Fig. 4.—Showing how the stability of absorbance at 390 mμ varies for different concentrations of *o*-benzoquinone in aqueous solutions containing catechol; temp. 26.4 ± 0.5°. The *o*-benzoquinone was produced in a 3-ml. volume of 0.1 *M* catechol solution (buffered to *p*H 5.1 with 0.1 *M* acetic acid–sodium acetate) by adding appropriate aliquots of 0.06 *M* ceric sulfate solution, 0.2 *M* in H_2SO_4, at zero time. The *p*H was readjusted to 5.1 by adding 0.16 ml. of 0.4 *M* sodium hydroxide for every 0.1 ml. of ceric sulfate solution used. The order of addition was catechol–buffer to ceric sulfate to sodium hydroxide.

Curve	$Ce(SO_4)_2$ soln., ml.	NaOH soln., ml.
1	0.30	0.48
2	.20	.32
3	.10	.16
4	.05	.08

can be seen that the quinone, as followed spectrophotometrically, disappeared much more rapidly in the system initially more concentrated in quinone (curve 1) than in the systems containing lower initial quinone concentrations (curves 2, 3 and 4). Furthermore, the absorbance at 390 mμ did not fall to zero because the products of the quinone–water–catechol reaction also absorbed in this same region. As a consequence, spectrophotometric measurements of *o*-benzoquinone stability, particularly in very dilute systems, may be misleading.

Regardless of the complications introduced spectrophotometrically by the products of the quinone removing reaction, it is apparent from Fig. 4 that this reaction will not manifest itself spectrophotometrically during the enzymatic oxidation of catechol until a significant concentration of quinone accumulates. As a consequence, the initial slope of the quinone production curve with time (Figs. 2 and 3) is roughly proportional to the rate of catechol oxidation. As the quinone-removing reaction picks up speed, however, it begins to tap off the quinone at an appreciable rate and a curvature in the spectrophotometric curve becomes evident. Shortly thereafter the quinone is removed as fast as it is formed and the quinone concentration in the system remains constant with time. This condition will persist as long as the enzyme remains sufficiently active to equal the quinone removing reaction.

Evidence in support of the above steady-state interpretation of the horizontal portion of the O. D. *vs.* time curve is given in Fig. 5. In this figure are

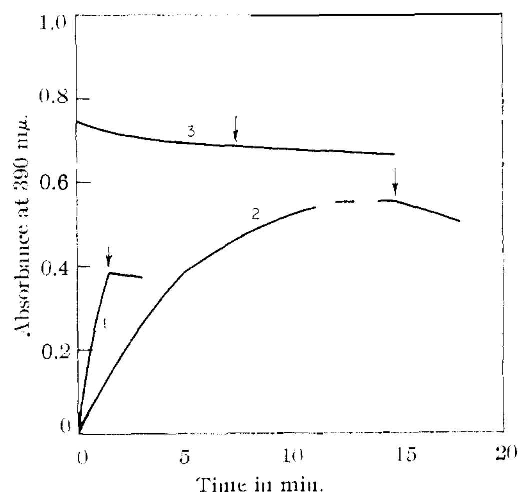

Fig. 5.—Showing the effect of cyanide on the course of color development in the tyrosinase-catechol reaction. Curve 1: a 3-ml. sample of 0.1 *M* catechol in 0.2 *M* acetate buffer was added at zero time to 0.1 ml. of enzyme in a 1 cm. spectrophotometer cell. At the 90 sec. point, 0.5 cc. of 0.018% aqueous KCN was added. Note that the enzyme was very rapidly inactivated and the quinone absorbance started to fall. Curve 2: same as curve 1 except less enzyme and the KCN was added after 15 minutes when the quinone absorbance curve became parallel to the time axis. The breaks in the curve correspond to shaking periods when the cell was removed from the recording spectrophotometer. Note that when the enzyme was inactivated by the KCN, the quinone absorbance curve started to fall, indicating that there was active enzyme present at the 15 minute period although the quinone production *apparently* had ceased. Curve 3: control experiment showing that KCN has no effect on the rate of disappearance of quinone produced by ceric sulfate oxidation of catechol. Oxidizing conditions similar to those of Curve 3, Fig. 4.

shown the results of experiments using KCN as an agent to rapidly inactivate the enzyme. In the experiment represented by curve 1, it can be seen

that KCN rapidly stopped the enzymatic production of quinone. In the absence of active enzyme, the unstable quinone then slowly disappeared from the system. In the case of curve 2, the addition of the same amount of KCN, after the O.D. *vs.* time curve had become horizontal, was also followed by a decrease in absorbance at 390 mμ. Since it is apparent from curve 3 that this amount of KCN had no effect on the fact of *o*-benzoquinone produced non-enzymatically, it is clear that the break in curve 2 was due only to the elimination of the enzymatic quinone-producing reaction. In other words, there must have been active enzyme producing quinone, and thereby maintaining a steady state, during the period that the O.D. *vs.* time curve (curve 2) was horizontal.

In the light of the foregoing results, the reactivation of color formation following the addition of ascorbic acid can be explained as a temporary smothering of the quinone-removing reaction (by lowering the quinone concentration to the point where its reaction with solvent is negligibly slow) so that the unaffected enzymatic quinone producing reaction becomes observable as an over-all rise in O.D.

When ascorbic acid is added initially to the enzyme–catechol reaction-mixture, it delays the appearance of the quinone color until it has all been oxidized. This reaction forms the basis of the chronometric method.[7] If, now, ascorbic acid here functions only as a reducing agent having no activating effect on the enzyme, the rate of browning in the solution, when the color does finally appear, should be equal (after correction for quinone loss) to the rate of color formation in a solution which is at the same stage of reaction but has had no ascorbic acid added to it.

In the experiments represented graphically in Fig. 6, varying amounts of ascorbic acid were added, at zero time, to several identical enzyme–catechol reaction systems and the eventual rate of color appearance recorded. If one compares the initial slopes of curves II, III, IV with the slope, at the same abscissa, of curve I (the blank), then it becomes apparent that Ponting's contention that ascorbic acid has an activating effect upon the enzyme, is not well founded. These results, obtained as they were during the first few minutes of reaction, constitute a good check on the validity of the chronometric method, and agree with the findings of Ingraham[15] in a recent investigation on the same subject.

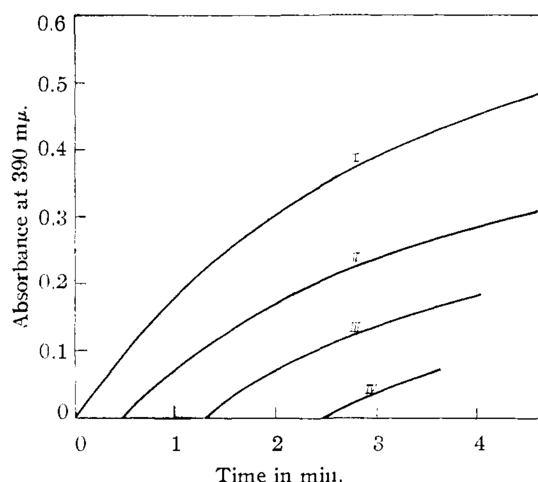

Fig. 6.—Showing the development of *o*-quinone color in tyrosinase-catechol solutions containing initially varying amounts of ascorbic acid; temp. 24.3 ± 0.5°.

Curve	1	2	3	4
Ascorbic acid, mg.	0	2.0	4.0	6.0

In each experiment a 10-ml. aliquot of a chronometric reaction mixture[6] (containing 10 ml. of 0.4 *M* phosphate–0.2 *M* nitrate buffer, *p*H 5.1, 1 ml. of 20 mg./ml. catechol solution, 0–3 ml. of 2 mg./ml. ascorbic acid solution, and copper-free water to a volume of 100 ml.) was pipetted into the special 1 cm. bubbler cell (Fig. 1) at zero time, 0.1 ml. of enzyme (3.3 units) was added and the air supply turned on. Note that the initial slopes of curves 2, 3 and 4 are equal to the slope of curve 1 at the same abscissa.

Acknowledgments.—The authors wish to express their thanks to Mr. Stanley Lewis for his painstaking efforts in preparing the enzyme. The financial assistance given by the Eli Lilly Company in support of this research is also greatly appreciated.

NEW YORK, N. Y.

(15) L. L. Ingraham, THIS JOURNAL, **78**, 5095 (1956).

[CONTRIBUTION FROM THE LABORATORY OF THE CHILDREN'S CANCER RESEARCH FOUNDATION]

High Molecular Weight Poly-α,L-glutamic Acid: Preparation and Optical Rotation Changes[1]

BY M. IDELSON[2] AND E. R. BLOUT[2]

RECEIVED JANUARY 31, 1958

The preparation of high molecular weight (degree of polymerization >500) poly-α,L-glutamic acid is described. The changes in optical rotation upon ionization of poly-α,L-glutamic acid in water were measured and found to be qualitatively similar to those which have been observed in the denaturation of some proteins.

High molecular weight water-soluble ionic polypeptides were desired in order to examine their physical-chemical and biological properties and to compare them with those of proteins. For these purposes a high molecular weight polypeptide is defined as one having a molecular weight around 50,000 or a degree of polymerization (*DP*) of at

(1) Polypeptides XXI. For the previous paper in this series see E. R. Blout and R. H. Karlson, THIS JOURNAL, **80**, 1259 (1958).

(2) Chemical Research Laboratory, Polaroid Corporation, Cambridge 39, Mass.