THE PREPARATION AND PROPERTIES OF HIGHLY PURIFIED ASCORBIC ACID OXIDASE

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The initial observation that certain plant tissues catalyze the aerobic oxidation of vitamin C (*l*-ascorbic acid) was made by Szent-Györgyi in 1928 (1). The existence in cabbage leaves of a specific enzyme "hexoxidase"—called "ascorbic acid oxidase" by later workers—was postulated by Szent-Györgyi (2) in 1930. Since that time numerous investigators (3-10) have described the preparation and properties of ascorbic acid oxidase from a variety of plant sources. Some of these preparations have been relatively impure, and a comparison of the properties of ascorbic acid oxidase from different sources is difficult, since it is our experience that the properties vary markedly with the degree of purity of the enzyme. As nearly as can be judged, the preparation described by Lovett-Janison and Nelson (10) from the yellow summer crook-neck squash (*C. pepocondensa*) appears to be the most highly purified of those reported to date. These investigators clearly showed that the enzyme was a copper-protein with a proportionality existing between the copper content and the enzyme activity. Their best preparation contained 0.15 per cent copper and had an activity of 430 units per microgram (γ) of copper and 630 units per mg. of dry weight. The enzyme in dilute solutions was colorless, but in concentrated solutions (about 8 to 10 mg. enzyme protein per cc.) was green. Intermediate concentrations of the enzyme were pale blue to greenish blue in color.

At about the same time Stotz (8) described an enzyme preparation from cucumbers which had a copper content as high as 0.25 per cent, and an activity of 850 units per mg. dry weight. At first glance it would appear that this preparation was considerably more pure than that of Lovett-Janison and Nelson. However, the activity unit used by Stotz is smaller by a factor of six, and a comparison of the preparations on the same unit basis[1] shows the preparation

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[1] The unit used by Stotz is based on an oxygen uptake of 100 mm.^{3} per hour during the enzymatic oxidation of ascorbic acid, while that of Lovett-Janison and Nelson is based on an oxygen uptake of 10 mm.^{3} per minute, or 600 mm.^{3} per hour. Converting the Stotz value of 850 units per mg. dry weight to Lovett-Janison and Nelson units gives a value of 141 units per mg. dry weight as compared to 630 units per mg. dry weight for the Lovett-Janison and Nelson preparation. Carrying the comparison still further, it can be seen that with the high value of copper reported by Stotz, the activity per γ of copper becomes very much lower than that reported by Lovett-Janison and Nelson; *i.e.*, 57 units per γ of copper as compared to 430 units per γ of copper.

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of Lovett-Janison and Nelson to be over four times more active per milligram dry weight than the preparation described by Stotz. Furthermore, it would seem that the latter preparation either contained considerable non-enzyme copper, or the proportionality factor between enzyme activity and copper is very much lower for cucumber enzyme than for the squash enzyme.

The purpose of this communication is to describe the preparation from summer crook-neck squash of an ascorbic acid oxidase solution having approximately twice the activity per milligram dry weight as that of Lovett-Janison and Nelson; and having a copper content of 0.24 per cent. Secondly, some of the more interesting properties of this highly purified preparation are compared with those of previously described preparations of lower purity.

Purification of Enzyme from Summer Squash

The procedure of Lovett-Janison and Nelson (10) for the preparation of the oxidase was followed with a few changes. Since these changes resulted in a purer preparation with a higher yield, the modified procedure is described:—[2]

Step I. Crude Juice.—Twenty bushels of yellow summer squash were peeled; the rinds were minced to a fine pulp and the pulp subjected to hydraulic pressure to remove all the juice. At this point 100 liters of crude juice were obtained, having a total oxidase activity of 4,500,000 units and an activity of 1.2 units per mg. dry weight. (For the definition and determination of ascorbic acid oxidase activity see (10). The pH of the crude juice was 5.9. A small amount of sodium tetraborate was added to bring the pH to 7.6. The crude juice was clarified by adding molar barium acetate (10 cc. per liter of juice) and the resulting suspension was allowed to settle overnight before syphoning off the supernatant liquid. This operation removed considerable color and some extraneous protein, without causing any appreciable loss of oxidase activity.

Step II. Fractionation by Ammonium Sulfate[3].—Barium ion was removed from the clarified juice by adding $(NH_4)_2SO_4$ to 0.3 saturation at room temperature (210 gm. per liter of juice). The precipitate was filtered off immediately. The enzyme was next precipitated from the filtrate by more $(NH_4)_2SO_4$ to 0.6 saturation and the precipitate was dissolved in about 9 liters of $M/15$ Na_2HPO_4 solution. This solution contained 4,390,000 units or 97 per cent of the enzyme as compared to 85 per cent of the enzyme at this point in the Lovett-Janison and Nelson procedure. Activity of the preparation was 88 units per mg. of dry weight.

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[2] Chemicals used in the enzyme preparation were: Barium acetate, $Ba(C_2H_3O_2)_2 \cdot 2H_2O$, Eimer and Amend, technical grade; ammonium sulfate, $(NH_4)_2SO_4$, General Chemical, granulated white grade; disodium phosphate, $Na_2HPO_4 \cdot 12H_2O$, General Chemical, A.C.S. specifications; magnesium sulfate, $MgSO_4 \cdot 7H_2O$, General Chemical, technical grade; lead acetate, $Pb(C_2H_3O_2)_2 \cdot 3H_2O$, Eimer and Amend, c. p. grade; and litharge, PbO, General Chemical, c. p. grade.

[3] All filtrations except those involving the lead-acetone precipitates were filtered with suction through a ¼ to ½ inch pad of celite (Johns-Manville No. 535). For the lead precipitates a ¼ inch pad of standard super-cel (Johns-Manville) was used.

Step III. Fractionation by Magnesium Sulfate.—The protein in the above solution was fractionated by the use of magnesium sulfate. The first fraction was precipitated by the addition of 500 gm. of magnesium sulfate per liter of solution, and contained enzyme along with inactive protein. However, in accordance with the experience of Lovett-Janison and Nelson, this precipitate could not be easily purified much further and was therefore discarded. More magnesium sulfate was added to the filtrate (about 400 gm. per liter) and the resulting precipitate contained the rest of the oxidase. The precipitate was filtered and dissolved in about $2\frac{1}{2}$ liters of $M/15$ Na_2HPO_4 solution. After dialyzing against running tap water for 2 days (temperature about 15°C.) the volume of the solution increased to about 3 liters. The solution contained 3,170,000 units or 70 per cent of the original activity and had an activity of 150 units per mg. dry weight.

Step IV. Fractionation by Alumina Adsorption.—The solution above was next treated with alumina reagent (11) (5 cc. of alumina per 100 cc. of solution) and filtered immediately. The alumina adsorbate, heavy with extraneous protein, was discarded although it also had considerable oxidase activity. The filtrate (now of a volume of 3150 cc. and containing 2,160,000 units) was treated with 0.1 saturated lead subacetate solution (1 cc. per 600 cc. of filtrate) to precipitate more extraneous protein before adsorbing the major portion of the enzyme to alumina. This was accomplished by adding alumina reagent (10 cc. per 100 cc. of solution) to the filtrate from the lead precipitation. The adsorbate was filtered off immediately, eluted with $M/15$ Na_2HPO_4 solution, and dialyzed overnight against running tap water (temperature about 15°C.). At this point the volume of the solution was 1500 cc., and contained 1,200,000 units or 27 per cent of the original activity. The solution had an activity of 232 units per mg. dry weight.

Step V. Fractionation by Lead Subacetate[4].—To continue the purification, each 100 cc. of the above solution was treated with 10 cc. of dry-ice cooled acetone and then 1 cc. of 0.1 saturated lead subacetate solution. This first lead precipitation was heavy and contained considerable enzyme; however, it was discarded since experience had shown that the enzyme could not be easily separated from the extraneous protein in the precipitate. A second precipitate was obtained by treating the filtrate with 5 cc. more of the 0.1 saturated lead subacetate solution per 100 cc. of filtrate after adding cold acetone as before (10 cc. per 100 cc. of filtrate). The precipitate was filtered off and saved.[5] The filtrate from this step was again treated with cold acetone as before and then with 15 cc. of the 0.1 saturated lead subacetate solution per 100 cc. of filtrate

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[4] The lead subacetate solution was prepared as follows: 420 gm. of $Pb(C_2H_3O_2)_2 \cdot 3H_2O$ and 140 gm. of PbO were ground in a mortar and mixed with 1400 cc. of water in a glass-stoppered bottle. After thorough shaking, it was allowed to stand for a week at room temperature and then filtered. One volume of the filtrate was diluted to ten volumes with water to make 0.1 saturated lead subacetate solution.

[5] The second lead acetate precipitate was dissolved in $M/15$ Na_2HPO_4 solution, which upon dialysis for 2 days, yielded a blue-green precipitate. The precipitate was redissolved in 10 cc. of 0.1 M (Cu^{++}-free) Na_2HPO_4 solution to give a blue-green solution having an activity of 5750 units per cc. Calculations indicated this solution to have an activity of about 640 units per mg. dry weight of protein material. No measurements of its copper content were made.

and again filtered. The precipitate was small and, although it contained about 100,000 units of activity, it was discarded as attempts at further purification failed. Further treatment of the filtrate with acetone lead subacetate failed to produce precipitation.

Step VI. Adsorption to Alumina and Dialysis.—The filtrate from the lead acetate treatment was highly active (total activity, 350,000 units) and was treated with alumina reagent (5 cc. per 100 cc. of solution). This operation was performed immediately in order to remove the active enzyme from the solution; thus preventing its inactivation by the Pb ion in the solution. All the activity was adsorbed to the alumina, which was filtered off and eluted with 130 cc. of $\text{M}/15$ Na_2HPO_4 solution. Upon dialysis for 2 days against redistilled water (Cu^{++}-free) at pH 6.5–7.0, an amorphous dark colored protein-like precipitate was observed in the dialyzing bag. This precipitate was filtered off by means of a porcelain Gooch filter and found to have a brilliant bluish-green color. The filtrate was active but contained also most of the non-active protein as shown by the fact that the activity per milligram dry weight was low; *i.e.*, 141 units per mg. The bluish-green precipitate was dissolved in 50 cc. of (Cu^{++}-free) 0.1 M Na_2HPO_4 (see preparation of Cu^{++}-free buffer solutions in legend of Fig. 1) to yield a highly active pale blue solution, containing 1970 units of enzyme activity per cc. and about 4.5 γ of Cu per cc. The blue color was comparable to that of a $Cu(NO_3)_2$ solution containing 2500 γ Cu^{++} per cc. The degree of purification effected by the precipitation during dialysis is shown by the fact that the blue solution had an activity of 1060 units per mg. dry weight. The pale blue solution contained a total of 88,500 units for a yield of 1.9 per cent of the original press juice activity. It is significant to note that these activity measurements were made in the manner prescribed by Lovett-Janison and Nelson (10). The dry weight and copper data on this solution, referred to as No. 15-OI in Table I, were obtained by further dialysis of a small sample for 2 days at pH 7.5 against frequent changes of redistilled Cu^{++}-free water. The dry weights of all solutions were determined by the method described by Lutz and Nelson (12), with the following modification; it was found unnecessary to seal off the dry weight bulbs after complete evaporation of the protein solution, because of the non-hygroscopic nature of this protein.

Copper Determinations

The method used for the copper determinations was that of Warburg and Krebs (13). To insure no contamination by extraneous copper, all samples were dialyzed, before making the copper determination, against distilled water which had been redistilled in Pyrex glass vessels.

Determination of Peroxidase Content of Purified Ascorbic Acid Oxidase

The method used for the determination of peroxidase was that of Balls and Hale (14). The purified oxidase preparation 15-OI was found to contain 0.000008 units of peroxidase per unit of ascorbic acid oxidase which compares favorably with a ratio of 0.0000078 obtained by Lovett-Janison and Nelson (10). The ratio in the crude squash juice was about 0.0022. The

preparation can be considered to be peroxidase-free, in view of the fact that in dilutions used to determine the ascorbic acid oxidase activity no peroxidase activity could be detected. Numerous authors (4, 10, 15) have reported that peroxidase activity accompanies ascorbic acid oxidase in their preparations; nevertheless the latter activity is independent of the former (15).

Determination of Catalase Content of Purified Ascorbic Acid Oxidase

Huszák (4) has reported a high concentration of catalase activity in his partially purified ascorbic acid oxidase preparation. However, the catalase activity in preparation 15-OI was found to be small. The method used for the determination of catalase activity was that of von Euler and Josephson (16).

TABLE I

Comparison of Several Ascorbic Acid Oxidase Preparations

Preparation No.	Concentration units/cc.	Units/γ Cu	Per cent Cu	Units/mg. dry weight
14-3A1...................	594	456	0.11	510
14-IIB3B2-A..............	675	407	0.15	592
14-IIB3C2................	600	400	0.15	588
11-L.J.*.................	531	406	0.15	612
12-L.J.*.................	516	490	0.15	631
15-OI...................	1970	450	0.24	1060
		Ave......... 428 ± 25‡		
15-OI§..................	3125	715	0.24	1680

* Best preparations obtained by Lovett-Janison and Nelson.

‡ Lovett-Janison and Nelson preparations and the modified value (715) are not included in the calculation of the average.

§ Activity measured after modified dilution procedure.

A Kat. f. value of 204 was obtained when the catalase activity was measured at an enzyme dilution of 1:25 (k_0 = 0.016; dry weight = 1.86 mg. per cc.). No catalase activity was detectable in preparation 15-OI when it was diluted sufficiently to determine ascorbic acid oxidase activity.

Comparison of Ascorbic Acid Oxidase Preparations

Several ascorbic acid oxidase preparations were made by the Lovett-Janison and Nelson procedure (10). The data showing the relative purity of each preparation are given in Table I. The two best preparations of Lovett-Janison and Nelson are included for comparison. The proportionality of enzyme activity to copper content as shown in Table I (428 ± 25 units per γ of copper) was obtained when the activities were measured under the conditions described by the above workers (10). This value is in excellent agreement with their original value; i.e., 430 ± 30 units per γ of copper.

For reasons which will become apparent below, it was deemed advisable to alter slightly the conditions of activity measurement from those originally described (10) since the altered procedure for measurement gave a considerably higher activity for a given amount of the enzyme. This alteration consisted of making up the enzyme dilution with a dilute solution of the inert protein gelatin rather than adding the water-diluted enzyme to the gelatin at the initiation of the measurement. The increase in activity effected by this modified dilution procedure can be seen by inspection of the values in the bottom line of Table I.

It was found that these highly purified enzyme solutions could be stored in the refrigerator for several months with little or no loss of activity, provided the enzyme concentration was in excess of 1 mg. per cc., the solution was sterilized with a few drops of highly purified toluene, and the solution was kept about 0.1 M in Na_2HPO_4. Such preparations, if dialyzed and stored in the absence of Na_2HPO_4, were unstable in that they showed a tendency to precipitate the activity in the form of an amorphous greenish-blue protein.

The Use of Gelatin in Ascorbic Acid Oxidase Activity Measurements

Adams and Nelson (17) observed that the addition of the inert protein gelatin to the reaction mixture, when measuring the activity of the enzyme tyrosinase, furnished protection to the enzyme against inactivation. Lovett-Janison and Nelson made the same observation in connection with the ascorbic acid-ascorbic acid oxidase system. As a consequence, they recommended that during the activity measurement of ascorbic acid oxidase, 5 mg. of gelatin be placed in the main part of the Warburg respirometer flask, to stabilize the enzyme against inactivation during the oxidation of the ascorbic acid. Later studies by Miller and Dawson (18) on tyrosinase systems, showed that the protective action of gelatin was variable, depending on the type of enzyme and the environmental conditions during measurement. As a consequence of these findings, it seemed advisable to study in more detail the effect of the inert protein gelatin on the activity and inactivation of the highly purified ascorbic acid oxidase preparation 15-OI.

The data given in Fig. 1 show the effect of gelatin on the action of highly purified ascorbic acid oxidase during the aerobic oxidation of ascorbic acid. The experiments were performed at pH 5.6 using the same amount of enzyme and ascorbic acid in each case but in one of the three experiments no gelatin was present in the reaction system. For further experimental details see the legend of the figure. As can be seen from curve I of this figure, when no gelatin is present in the reaction system, the enzyme becomes inactivated very rapidly and the enzymatic oxidation ceases before the ascorbic acid is completely oxidized. The reproducibility of the activity measurements (rate of oxygen uptake per minute) is poor without the use of gelatin; comparable manometer

readings in two simultaneous runs varying as much as 20 to 25 per cent from one another. It is of interest to note (curve II) that with this preparation of the enzyme little or no protective action was observed when gelatin was used in the reaction mixture in the way prescribed by Lovett-Janison and Nelson—namely, the diluted enzyme is in the side arm during the temperature equilibration period and is then added to the reaction mixture containing gelatin at zero time. In the case of curve III, the reaction mixture contained the same amount of enzyme and less gelatin than in the case of curve II. However, the enzyme dilution was made with a dilute gelatin solution, rather than with water. Thus in this case (curve III) the enzyme was in a protein environment at all times during the process of dilution and temperature equilibration. A comparison of the initial rates of oxidation (curve III/curve I or II = 2.5/1.6 units in reaction flask) makes it apparent that much of the gelatin effect is one of protection against loss of enzyme activity occurring prior to the activity measurements; *i.e.*, the period of time between making the dilution and starting the measurement. It was found that this method of diluting the enzyme with gelatin did not change the range of concentration of enzyme over which there is a direct proportionality between rate of oxidation of ascorbic acid and the amount of enzyme used in the reaction flask. The most suitable range for measuring the activity was found to be from about 1 to 2.5 units of enzyme in the reaction flask under optimum conditions of pH and substrate concentration.

The loss of enzyme activity, when highly purified enzyme preparations are highly diluted, has been observed in several systems. Saul and Nelson (19) noticed a loss in activity when highly purified yeast invertase preparations were diluted. This abnormal decrease in activity almost vanished, when a little gelatin was added to the enzyme solution, either before or after dilution. Adams and Nelson (17) found, with highly purified tyrosinase preparations, a similar loss of activity upon dilution which was apparently reversed by using gelatin in the reaction system during the activity measurements. In the case of the highly purified preparation of ascorbic acid oxidase (15-OI) one notes that whereas a marked increase in activity is obtained when small amounts of gelatin are added to the enzyme during dilution, no such increase is observed when the diluted ascorbic acid oxidase is added at zero time to the reaction mixture containing gelatin. In other words, apparently gelatin cannot reverse the loss of enzyme activity occurring during the 15 minute temperature equilibration period. Experiments designed to learn more of this phenomenon were carried out and the results are discussed below.

To determine the effect of gelatin concentration, experiments were performed in which the concentration of gelatin in the 1 cc. of enzyme added to the reaction mixture was increased by several increments from zero to 5.5 mg. of gelatin per cc. The results are shown in Fig. 2. It will be seen that the rate of oxidation of ascorbic acid for a given amount of the enzyme-protein rapidly increases with
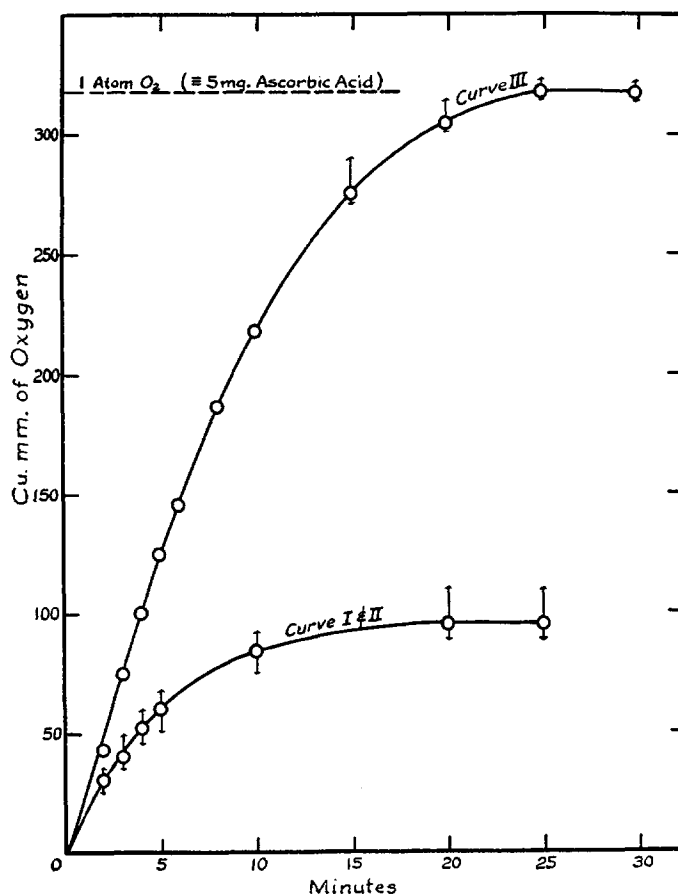
FIG. 1. Curves showing the effect of gelatin on the activity and inactivation of highly purified ascorbic acid oxidase.

Curve I. Reaction Mixture.—1 cc. of enzyme solution 15-OI (diluted 1:1250 with water as described below) placed in side arm of 50 cc. Warburg respirometer flask. Main part of flask contained 2 cc. of 0.1 M citric acid-0.2 M Na₂HPO₄ buffer, pH 5.7, 1 cc. of *l*-ascorbic acid solution (5 mg. per cc.) made up freshly each day in Cu⁺⁺-free water, and water to make a total reaction volume of 8 cc. pH of reaction mixture 5.6. Generally at least five Warburg manometers set up in identical fashion for each experiment. The flasks and contents were equilibrated in the thermostat at 25°C. for 15 minutes before initiating the reaction. Manometers were not shaken during this period in order to minimize surface denaturation of the enzyme-protein. During the run, the manometers were shaken at a rate of 120 oscillations per minute. Initial reading of oxygen uptake made at 2 minutes.

Curve II. Reaction Mixture.—Same as curve I, except 1 cc. of gelatin solution (5 mg.) substituted for 1 cc. of water in main reaction compartment.

increase in gelatin concentration up to 0.05 mg. of gelatin in the 1 cc. of enzyme. Very little difference in the initial rate of oxidation is effected by the use of more gelatin, although the optimum per cubic centimeter of enzyme appears to be in the vicinity of 0.5 mg. of gelatin. With much larger amounts of gelatin per cubic centimeter of enzyme, such as 5.5 mg., the initial rate of oxidation is a little lower and the reaction course is characterized by an initial lag period; *i.e.*, probably such large amounts of gelatin affect the initial enzyme-substrate relationship. In all subsequent work with this enzyme preparation in which gelatin was added during the enzyme dilution, the optimum concentration of 0.5 mg. of gelatin per cc. of enzyme solution was used.

The loss in activity of the enzyme, occurring when the enzyme is allowed to stand for a period in solutions of very low concentration, is undoubtedly due to some type of denaturation or unfolding of the enzyme-protein into less active forms. Apparently the gelatin exerts its effect by hindering this type of change of the enzyme-protein. To throw more light on this phenomenon of an inert protein protecting a highly active protein in this way, and to get some estimate of the time factor involved, the following experiments were performed. Varying amounts of time were allowed to elapse between making the final dilution and measuring its activity. During this time the enzyme was maintained at approximately 25°C. Two series of dilutions were set up; both to a final ratio of 1:1250. In one series the enzyme was diluted using only water, and in the other using gelatin solution so that the final concentration of gelatin was 0.5

Curve III. Reaction Mixture.—Enzyme solution diluted from subdilution (see below) as follows: 1 cc. of gelatin (5 mg. per cc.) and 1 cc. of subdilution diluted to 10 cc. with refrigerated water. 1 cc. of this dilution placed in side arm of flask; all other reactants as in curve I.

Diluting the Enzyme.—The enzyme dilution (1:1250) was made as follows: 1 cc. of the enzyme solution 15-OI (see Table I) was diluted to 25 cc. with water. This dilution could be used as a master dilution without loss of activity for a period of about a week. 1 cc. of this master dilution was next diluted to 5 cc. with water. This subdilution was found to be stable for several hours. The final dilution was made by diluting 1 cc. of the subdilution to 10 cc. All dilutions were made with Cu^{++}-free water kept at refrigerator temperature (about 2-5°C.) and were immediately stored in the refrigerator until used.

Other Details.—The buffer was made from citric acid and Na_2HPO_4, each thrice recrystallized from distilled water which had been redistilled from all-glass apparatus. All Warburg flasks were cleaned with fresh dichromate cleaning mixture, rinsed with distilled water, and steamed out for 15 minutes prior to use. One flask containing all reactants except enzyme, was used as a blank in all runs. This served as a barometric control and also as a blank for ascorbic acid oxidation not due to enzymic oxidation. The latter was always found to be negligible during the time of reaction when the above cleaning precautions were observed. Arrows on the individual points of the curves represent maximum deviations from the average of several runs.

mg. per cc. of enzyme. The results are shown in Table II. It is apparent that the loss in activity is a function of time after making the final dilution, and that
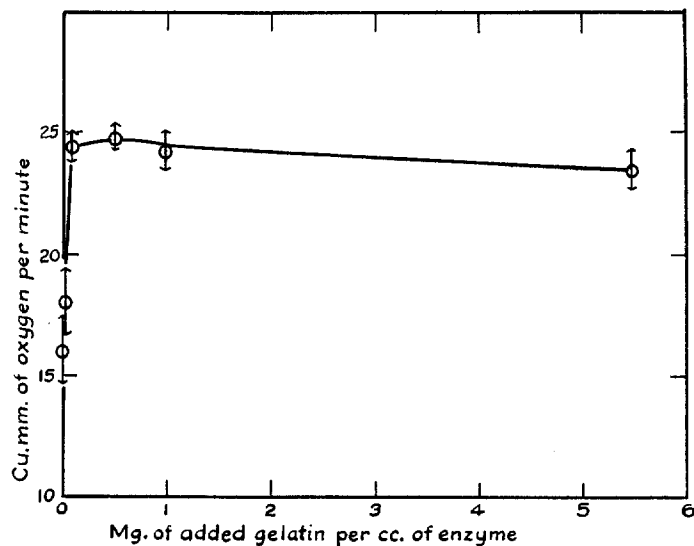

FIG. 2. Showing the effect of gelatin concentration on the initial rate of oxidation of ascorbic acid by a given amount of highly purified ascorbic acid oxidase. Gelatin added during dilution of enzyme.

1 cc. of enzyme (15-OI) diluted 1:1250 used for each rate determination. In making up the dilution, 1 cc. of the oxidase subdilution (described in legend of Fig. 1) was mixed with appropriate amounts of gelatin and diluted to a volume of 10 cc. with water kept at refrigerator temperature. In each rate determination 1 cc. of the gelatin-enzyme mixture was added from the side arm of the flask to initiate the reaction. Ascorbic acid, buffer, and other experimental details as described in legend of Fig. 1. The values of oxygen uptake per minute shown in the figure were obtained for the gelatin concentrations in excess of 0.5 mg. per cc. from the linear portion of curves showing the oxygen uptake as a function of time. In these cases little inactivation of the enzyme was evident within the first 5 minutes. For the lower concentrations of gelatin it was necessary to take the initial 2 minute reading as the initial rate of oxygen uptake. Each experimental point is the average of at least five determinations. Average deviations are represented by the arrows on each point.

the gelatin merely slows down the rate of denaturation or unfolding of the enzyme-protein.

pH-Optimum of Highly Purified Ascorbic Acid Oxidase

Various pH optima of ascorbic acid oxidase activity, measured in different buffer systems and with enzymes from various sources, have been reported by

a number of investigators. Using a citrate-phosphate buffer and a cucumber oxidase, Tauber, Kleiner, and Mishkind (3) reported an optimum lying between pH 5.56 and 5.93; Srinivasan (20) using drumstick oxidase reported pH 4.6 — 5.6, and Ghosh and Guha (21) reported a value of pH 5.6 for white gourd oxidase. In acetate buffer solutions, pH optimum values lying between 5.0 and 6.6 have been reported (3, 20, 21). While there seems to be little agreement in acetate buffers, all workers are fairly well agreed that in citrate-phosphate systems the optimum lies near pH 5.6 regardless of the source of the enzyme. This value has been substantiated using the highly purified summer squash oxidase (15-OI) (see Fig. 3). Since Steinman and Dawson (22)

TABLE II

Showing the Protective Effect of Gelatin upon the Stability of Diluted Ascorbic Acid Oxidase

Elapsed time between making dilution and initiating the reaction	Enzyme diluted* in water	Enzyme diluted in gelatin solution
	Activity/cc.	Activity/cc.
min.		
6	2.40 ±0.10 units	2.45 ±0.05 units
10	1.90 ±0.10 "	2.45 ±0.05 "
21	1.70 ±0.15 "	2.45 ±0.05 "
30	1.55 ±0.05 "	2.20 ±0.10 "
60	1.20 ±0.10 "	2.00 ±0.20 "

Rate determinations made using 1 cc. of enzyme (15-OI) diluted 1:1250 as described in legend of Fig. 1, (curves I and III) except that the freshly diluted enzyme was not placed in side arm of flask until after the 15 minute temperature equilibration period. Flasks were then quickly returned to thermostat and the reaction was initiated at 6 minutes, 10 minutes, etc., from the time the enzyme had been diluted. Room temperature about 25°C. The above activity values are the average of at least three determinations. Those in the gelatin series are based on linear portion of reaction course, whereas those in other (no gelatin) series are based on first 2 minute reading.

* No gelatin whatever added to Warburg flask.

have shown that the auto-oxidation of ascorbic acid in acetate buffers is considerable while in citrate buffer systems it is negligible, only the latter were used in this investigation. Although gelatin, used in the manner previously described, was found to decrease somewhat the sharpness of the pH optimum, the position of the optimum was not altered (compare curves I and II, Fig. 3). When the concentration of substrate was doubled (from 2.5 to 5 mg. of ascorbic acid in 8 cc. of reaction volume), no effect could be observed on the rate of oxidation or on the position or character of the pH optimum when the enzyme was diluted so as to contain 0.5 mg. of gelatin per cc.

Substrate Optimum Studies

Tauber, Kleiner, and Mishkind (3) have studied the effect of substrate concentration on the activity of cucumber ascorbic acid oxidase over a range of

1 to 5 mg. of substrate in a 7 cc. reaction volume and found that the activity of cucumber ascorbic acid oxidase was independent of the substrate concentration. Srinivasan (20) and Ebihara (7), using about the same range of variation of ascorbic acid, came to the same conclusion. The effect of larger concentrations
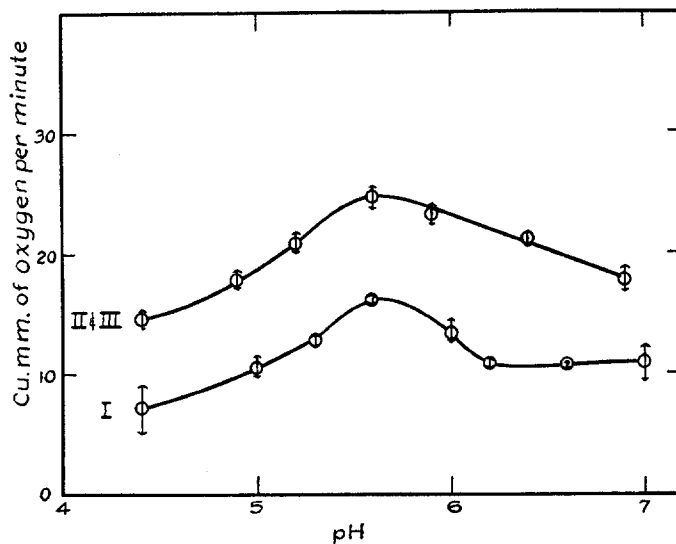

Fig. 3. Showing the effect of gelatin on the pH optimum of highly purified ascorbic acid oxidase.

Curve I. No Gelatin.—1 cc. of enzyme (15-OI) diluted (1:1250) as described in legend of Fig. 1. The 8.0 cc. reaction mixture contained 2.5 mg. of ascorbic acid and 2 cc. of 0.1 M citrate–0.2 M phosphate buffer (pH of mixture as shown). Rate measurements made as described in legend of Fig. 1.

Curve II. Gelatin.—1 cc. of enzyme (15-OI) diluted (1:1250) with gelatin as described in legend of Fig. 1 (curve III) (0. 5 mg. gelatin per cc. enzyme). Ascorbic acid and buffer as in curve I.

Curve III.—Same as curve II except 5 mg. of ascorbic acid used.

Each experimental point the average of at least three determinations. Average deviation represented by arrows on each point.

of substrate on the enzyme apparently has not been previously studied. As will be seen from Fig. 4, the effect of varying the initial substrate concentration is somewhat more evident in the absence of gelatin than in its presence. When no gelatin is present in the system, (curve I) a significant decrease in the original rate of oxidation is effected by using amounts of ascorbic acid in excess of 5 mg. per 8 cc. reaction volume. However, when gelatin is present (curve II) there is little difference in activity caused by a variation in ascorbic acid from 2.5 to 10 mg. The results indicate that although purified ascorbic acid oxidase has

no sharp substrate optimum, activity measurements by the usual respirometer method should be made with about 5 mg. of ascorbic acid per 8 cc. reaction volume. They also indicate how inert protein material, such as is present in impure enzyme preparations, may tend to mask substrate enzyme relationships.
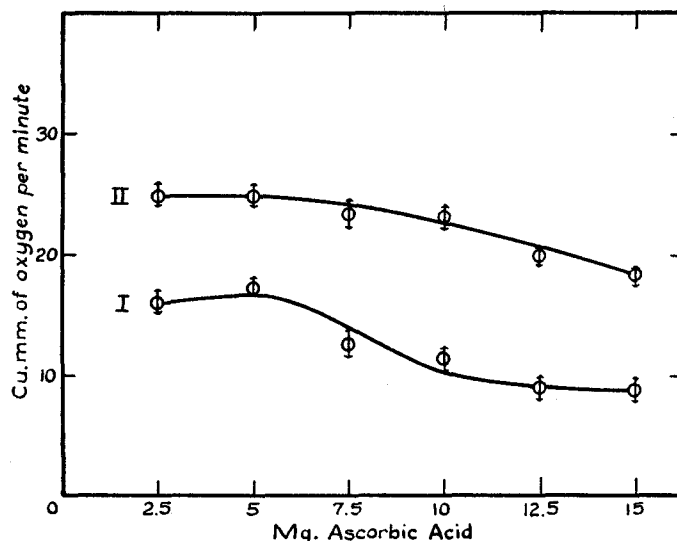

FIG. 4. Showing the effect of original substrate concentration on the activity of highly purified ascorbic acid oxidase.

Curve I. No Gelatin.—1 cc. of enzyme (15-OI) diluted with water. Measurements made at pH 5.6 as described in legend of Fig. 1.

Curve II. Gelatin.—1 cc. of enzyme (15-OI) diluted with gelatin as described in legend of Fig. 1 (curve III). All other conditions as in curve I.

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SUMMARY

1. A method is described for the preparation of a highly purified ascorbic acid oxidase containing 0.24 per cent copper.

2. Using comparable activity measurements, this oxidase is about one and a half times as active on a dry weight basis as the hitherto most highly purified preparation described by Lovett-Janison and Nelson. The latter contained 0.15 per cent copper.

3. The oxidase activity is proportional to the copper content and the proportionality factor is the same as that reported by Lovett-Janison and Nelson.

4. When dialyzed free of salt, the blue concentrated oxidase solutions precipitate a dark green-blue protein which carries the activity. This may be prevented by keeping the concentrated solutions about 0.1 M in Na₂HPO₄.

5. When highly diluted for activity measurements the oxidase rapidly loses activity (irreversibly) previous to the measurement, unless the dilution is made with a dilute inert protein (gelatin) solution. Therefore activity values obtained using such gelatin-stabilized dilute solutions of the oxidase run considerably higher than values obtained by the Lovett-Janison and Nelson technique.

6. The effect of pH and substrate concentration on the activity of the purified oxidase in the presence and absence of inert protein was studied.

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