ASCORBIC ACID (VITAMIN C) OXIDASE*

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Von Szent-Györgyi (2, 3) observed in 1930 that if a piece of cabbage leaf is placed in a respirometer in the presence of KOH it takes up oxygen. Mincing the cabbage decreased the intensity of this action. If ascorbic acid (at that time named by von Szent-Györgyi hexuronic acid) was added to the pulp, it was oxidized. This effect disappeared if the pulp was first boiled; hence he concluded it is an enzyme reaction. Von Szent-Györgyi (2) states:

"Study of the kinetics of this enzyme has clearly shown that this enzymic function is a complicated one and that the hexuronic acid is not immediately oxidized by the enzyme. The enzyme contains a special substance or grouping, 'x,' which is first oxidized by the oxygen. This oxidized 'x' then in turn oxidizes the hexuronic acid and is itself reduced again."

He was also of the opinion that the enzyme first brings about the oxidation of 2 labile hydrogen atoms believed to be present, since the structure of ascorbic acid had not been established at that time.

We have been able to isolate an extremely powerful enzyme from the pericarp of the Hubbard squash (Cucurbita maxima), which differs considerably from the one described by von Szent-Györgyi. It oxidizes ascorbic acid instantaneously and completely without interaction of other catalytic agents. Synthetic as well as natural l-ascorbic acid1 has been employed as substrate.

* A preliminary report of this paper has been read before the Society for Experimental Biology and Medicine (1).

1 We are greatly indebted to Professor von Szent-Györgyi for a sample of natural l-ascorbic acid and to Hoffman-La Roche, Inc. for generous samples of their synthetic l-ascorbic acid (redoxon).
The degree of oxidation was measured by the oxidation-reduction indicator, sodium 2,6-dichlorobenzenone indophenol (4). The indicator solution was made up fresh every 4 or 5 days, and was kept neutral. These two precautions are essential for accuracy. It must be remembered that ascorbic acid is autoxidizable and that proper controls must be made. Furthermore, its solutions are quite acid in reaction and, when employed in higher concentrations, neutralization of the acid is necessary even if a buffer is subsequently added.

EXPERIMENTAL

Preparation of Enzyme—The pericarp (edible portion) of ripe Hubbard squash was minced in a meat chopper. To 1 kilo of minced plant tissue 3 liters of 30 per cent alcohol were added and the mixture was gently shaken for 5 minutes in a 5 liter flask. The extract, which had a pH of 5.73, was centrifuged off, filtered, and an equal volume of acetone added. A yellow precipitate which separated on the surface of the fluid was removed with a glass rod, placed in an Erlenmeyer flask, and washed with acetone, which removed all the yellow pigment. The precipitate was dissolved in 400 cc. of distilled water and again precipitated with an equal volume of acetone. This procedure was repeated once more. The precipitate was then dried in a vacuum over H₂SO₄. The operations with acetone were performed quickly to avoid as much as possible any inactivation of the enzyme by the acetone.

This dry preparation does not immediately dissolve in distilled water but if left at room temperature for an hour or two with occasional shaking it does dissolve. It is 500 times as active as the original 30 per cent alcoholic extract, having an activity of 10,000 units per gm. The 30 per cent alcoholic extract had an activity of 2 units per cc. We suggest as a unit the amount of ascorbic acid oxidase which will oxidize 50 per cent of 0.5 mg. of ascorbic acid at 40⁰ in 5 minutes with 1 cc. of M Na acetate buffer of pH 5.6 in a total volume of 5 cc. Enzyme activity is stopped by the addition of 1 cc. of 2 per cent H₂SO₄ and the amount of unoxidized ascorbic acid titrated with the oxidation-reduction indicator, sodium 2,6-dichlorobenzenone indophenol. We dissolved 100 mg. of this dye in 250 cc. of boiling water and filtered it; this solution was standardized with crystalline l-ascorbic acid. The
colorimetric ferricyanide method may also be used to estimate ascorbic acid (5).

Aqueous solutions of the enzyme as well as dialyzed 30 per cent alcoholic extracts keep well if kept in a refrigerator. 18 hours dialysis involves no loss in activity of the extract. The yellow pigment which is removed by the acetone washings has no effect on the pigment-free enzyme. Thus it appears that the ascorbic acid oxidase is not related to the flavin oxidase of Warburg and Christian (6).

*Specificity for Ascorbic Acid*—The ascorbic acid oxidase oxidizes ascorbic acid probably by introducing two OH groups at the double bond (see accompanying formulas). The hydrogen thus liberated combines with atmospheric oxygen, the latter playing the rôle of a hydrogen acceptor. If oxygen is excluded by performing the experiment in an atmosphere of N₂, no oxidation of the ascorbic acid by the enzyme takes place. After the reduced form has been oxidized by the oxidase it may be reduced by H₂S, and when the H₂S is removed by N₂ it possesses the original reducing power.

In contrast with dialyzed cabbage leaf extracts the dialyzed squash extract as well as solutions of the purified enzyme does not give any of the color reactions which certain other oxidases give; e.g., benzidene, guaiacol, pyrogallol, catechol, phloroglucinol, resorcinol, naphthoresorcinol, vanillin. Nor does the ascorbic acid oxidase affect glutathione, cysteine, tyrosine, adrenalin, or glucose boiled with alkali.
Ascorbic Acid Oxidase

Presence of Single Enzyme—Von Szent-Györgyi states that the hexuronic acid oxidase of cabbage leaves is not a single enzyme but apparently involves a number of catalysts in a complicated mechanism. A study of the kinetics of the oxidase of the Hubbard

![Graph 1](image1.png)

**Fig. 1.** Influence of increasing amounts of substrate. The experiment was performed with the following. 2 cc. of buffer, pH 5.83 (0.15 M phosphate-citrate buffer), 1 cc. of enzyme solution, total volume 7 cc., temperature 20°. The mixture was shaken in 400 cc. beakers for 20 minutes.

![Graph 2](image2.png)

**Fig. 2.** Influence of increasing amounts of enzyme. The experiment was performed with the following. 1 cc. of buffer, pH 5.93 (0.15 M phosphate-citrate buffer), 1 cc. of ascorbic acid solution (0.5 mg. of redoxon), total volume in each case 4 cc., temperature 40°, time 5 minutes.
squash, however, indicates that here we are dealing with a single enzyme. Fig. 1 shows that a constant quantity of enzyme produces a constant amount of oxidation with increasing quantities of substrate under the conditions of the experiment. This harmonizes with the Michaelis-Menten theory. From Fig. 2 it appears that the catalytic action is directly proportional to the amount of enzyme used, the two experiments corroborating each other.

### Table I

*Optimum pH of Ascorbic Acid Oxidase*

In both experiments the following were used: 1 cc. of buffer, 0.5 mg. of neutralized ascorbic acid (redoxon), 2 cc. of enzyme, total volume 4 cc., temperature 40°, time 5 minutes.

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<th>Oxidation per cent</th>
<th>pH</th>
<th>Oxidation per cent</th>
</tr>
</thead>
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<td>66</td>
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</table>

*With 0.1 M glycine optimum pH values similar to those obtained with the M sodium acetate buffer were obtained.

Effect of pH—The optimum pH of the ascorbic acid oxidase is between 5.56 and 5.93 when 0.15 M phosphate-citrate buffer was added to neutralized ascorbic acid (Table I). If, however, M Na acetate buffer was employed, the optimum pH was between 5.38 and 5.57. The pH of squash juice is 5.47.

The ascorbic acid oxidase is only slightly active below pH 4.0 and above 7.0, thus having a very limited range of activity, restricted to the acid side of the pH scale. The enzyme, however, is more easily destroyed by an excess of H ions than of OH ions.
Ascorbic Acid Oxidase

If a solution of the enzyme is titrated to pH 2.0 with HCl and kept at 22° for 15 minutes, it becomes completely inactive. However, if it is adjusted to pH 10.0 with NaOH, there is very little inactivation under similar conditions. Its stability at such a definitely alkaline pH is remarkable since some other oxidases, e.g. milk peroxidase (7), are completely destroyed under these conditions.

The ascorbic acid oxidase is remarkably stable to O₂ (aeration), to CO₂, and to small amounts of KCN (0.005 per cent). Large amounts of KCN (0.01 per cent) inhibit and hydrogen sulfide, an activator of many enzymes, irreversibly inactivates this oxidase.

Chemical Nature of Ascorbic Acid Oxidase—The only protein color test a solution of 1 mg. of enzyme per cc. gives is the xanthoproteic. The dry substance gives a biuret test and a Millon’s test and an odor of burning hair when heated on a platinum foil. It gives a very strong Molisch reaction, due to large amounts of a polysaccharide accompanying the enzyme, which we have been unable to remove thus far. Saturated solutions of neutral salts do not give a precipitate when added to the dialyzed 30 per cent alcoholic extract or to the solution of the dry enzyme (1 mg. per cc.), nor do other protein precipitants. This is quite the opposite to the behavior of the cabbage oxidase. Acetone, however, precipitates it, and alcohol has a very destructive effect.

Inactivation of Ascorbic Acid Oxidase by Trypsin—Trypsin inactivates the ascorbic acid oxidase. Table II summarizes an experiment in which a 0.1 per cent trypsin (Fairchild Brothers and Foster) solution was employed. At pH 6.4 (0.1 M phosphate

<table>
<thead>
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<th>Digestion time</th>
<th>Inactivation</th>
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<tr>
<td>hrs.</td>
<td>per cent</td>
<td>hrs.</td>
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buffer) there was 23 per cent inactivation in 72 hours at 37°. If, however, a 0.5 per cent trypsin solution at pH 6.9 (0.1 M phosphate-citrate buffer) is used, the inactivation is much more rapid and in 72 hours 48 per cent of the enzyme is digested. The digests contained 5 cc. of buffer, 5 cc. of enzyme solution (5 units per cc.), and 5 cc. of trypsin solution. Controls with boiled trypsin solutions were run. Toluene was used as a preservative. The pH was controlled by the electrometric method except in the case of solutions containing ascorbic acid, which were determined colorimetrically.

SUMMARY

Our enzyme differs in various ways from the hexoxidase which von Szent-Györgyi found in cabbage leaves. The hexoxidase oxidizes not more than 25 per cent of the substrate even when present in excess, and its kinetics, as shown by von Szent-Györgyi, point to a very complicated effect of a number of catalysts. The enzyme of the Hubbard squash oxidizes 100 per cent of the substrate rapidly and its kinetics are those of a single enzyme. It does not affect phenols, glutathione, cysteine, or adrenalin, nor could we find any other substrate for this enzyme. Because of this, it seems to be a specific enzyme which we wish to name ascorbic acid oxidase.

The oxidase is inactivated by trypsin, which shows that like many other enzymes (8), it is either a protein or has protein as an indispensable part of it. This must be the case even though it gives negative or very weakly positive protein reactions. That enzyme or protein solutions may be too dilute to respond to protein precipitants and color tests is probable. The diluting agent may be a polysaccharide or a lipoidal substance. Conclusions concerning its chemical nature cannot be drawn from such solutions. The digestion by trypsin, however, is very significant.

The rôle of this enzyme in the physiology of the plant is at present obscure. There is practically no ascorbic acid, reduced or oxidized, present in the fruit. It is possible that it plays a part in the early life processes during ripening or that it is stored in this organ for use by the leaves.
Ascorbic Acid Oxidase

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