80. ASCORBIC ACID OXIDASE FROM CUCUMBER

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THE oxidation of ascorbic acid by plant tissues (*in vitro*) was observed by Sz. Györgi [1928] who later postulated an enzyme responsible for this oxidation which might be connected with the function of the vitamin *in vivo*. The same general phenomenon was observed by many other investigators who used widely different plant tissues, and the dynamics of the reaction and its relationship to glutathione were worked out by Hopkins & Morgan [1936] who used the pressed juice from cauliflower as a source of the enzyme.

A certain amount of confusion has existed, however, since Barron *et al.* [1935] showed that ascorbic acid was not autoxidizable at pH below 7.6 and that the oxidation was catalysed by minute traces of Cu present in the solutions as impurity. Examination of the Cu content of vegetable juices showed that the oxidation of ascorbic acid by these juices could be accounted for mainly by the Cu. Haemochromogens also act as catalyst for this oxidation. McCarthy *et al.* [1939] further showed that the so-called natural enzyme activity could be imitated by loose combinations of Cu ions and protein and suggested that the vegetable juices, which contain both Cu and protein, oxidized ascorbic acid by virtue of the mutual presence of these two factors and, consequently, there was no need to postulate the existence of a true ascorbic acid oxidase. It should be noted, however, that the amounts of Cu present in the synthetic 'enzymes' reported by these authors were in excess of the amounts of Cu in the ionic form required to catalyse the reaction at the same rate under similar conditions: in other words, protein, while imposing upon the Cu ions the qualities of an enzyme, hindered rather than accelerated the catalysis. As will be shown later, ascorbic acid oxidase is much more active catalytically than the same amount of Cu in the ionic form. The Cu in the synthetic enzymes, moreover, was all removed by dialysis against water, a treatment which only removed part of the Cu from vegetable juices. A certain amount of the catalytic oxidation of ascorbic acid by plant juices is probably due to the presence of Cu ions but, as Spruyt & Vogelsgan [1938] have pointed out, this is insufficient completely to account for the oxidase activity.

Kubowitz [1938] isolated from potato an enzyme which oxidized polyphenols and which, in the pure state, contained some 0.2% Cu which was removed by dialysis against dilute acid but not water. Kubowitz also succeeded in reconstituting the enzyme 'molecule' by adding to a Cu-free, inactive enzyme preparation an inorganic Cu salt. More recently, Lovett-Janison & Nelson [1940] isolated from crook-neck squash an enzyme which contained 0.15% Cu, was free from peroxidase activity, and which catalysed the oxidation of ascorbic acid to a marked degree without showing significant activity towards phenols. The method used by these authors to express the activities of their preparations makes it difficult to find a suitable basis for comparison with the oxidase from

cucumber described here, so that no opinion can be advanced about the possible identity of the two enzymes. Both, however, show great catalytic activity in the oxidation of ascorbic acid, both contain Cu in amounts related to these activities, and both fail to catalyse the oxidation of polyphenols to a significant extent.

The experiments described here provide evidence for the existence in cucumber tissue of a discrete ascorbic acid oxidase in which the prosthetic group is Cu.

EXPERIMENTAL

The reaction rate for the oxidation of ascorbic acid was measured in the Warburg manometric apparatus using reaction vessels of the single side-bulb type. The reaction flasks each contained 4.20 ml. liquid: 0.20 ml. 10 % KOH in the centre cup with filter paper; 0.20 ml. ascorbic acid solution containing 25 μ -mol. in the side bulb; and 3.80 ml. in the main space. 2.00 ml. buffer solution, 1.00 ml. portions of enzyme preparation, 0.20 ml. portions of suitable dilutions of other reagents such as diethyldithiocarbamate or CuSO_4 were used and the volume in the main space was made up to 3.80 ml. with distilled water. The buffer solutions used were acetate (M), McIlvaine's phosphate-citrate ($M/10$) and Clarke's phosphate ($M/4$), all at pH 6.0, the accuracy being tested in the Beckmann pH meter.

The reaction was started by tipping the ascorbic acid solution into the main space after temperature equilibration had been reached at 25°. Reaction rates were calculated from the initial, linear O_2 uptakes. The levels of the manometers were read every 5 min. after tipping except in the case of extremely small O_2 uptakes when the time interval was suitably increased. Control flasks containing (1) ascorbic acid and buffer solution, and (2) all reagents except ascorbic acid, were set up each time and the pH of the contents of each flask was checked after the reaction had been completed.

Cu-free distilled water was prepared by distilling water obtained from a laboratory electric still successively from $\text{K}_4\text{Fe}(\text{CN})_3 + \text{Ba}(\text{OH})_2$, and $\text{KMnO}_4 + \text{Ba}(\text{OH})_2$. The distillate was then distilled several times from an all-glass pyrex still incorporating a 12-bulb fractionating column until a test run with reagents prepared from this water showed an O_2 uptake of less than 5 $\mu\text{l.}$ per hr. with 25 μ -mol. of ascorbic acid at pH 6.0, in acetate buffer. Acetate buffer was used for the test since with it no inhibition of Cu catalysis occurs, as it does slightly with phosphate buffer. Similar precautions were taken with all reagents employed: ascorbic acid solutions prepared under these conditions were quite stable and the titre remained constant over long periods of time.

Cucumber juice was prepared by autolysing cucumber tissue. A cucumber was frozen overnight in a freezing mixture in the ice-chest before being sliced. The portions were placed in the neck of a large filter funnel above a porcelain grid or a loose plug of cotton wool and were allowed to thaw; the juice was collected in a pyrex bottle and stored in the ice chest. This method of preparation yielded a larger volume of clearer juice than any other tried, and avoided the necessity of further treatment to remove extraneous matter.

Dialysis of the juice was carried out in cellophane sacs of unknown porosity against Cu-free water at 0° and every care was taken to prevent contamination of the reagents. The dialysate was changed every 24 hr. and the ratio of the volume of cucumber juice to the volume of dialysate was kept constant at 1 : 4 since the detection and determination of Cu dialysed from the juice by means of the rate of catalytic oxidation of ascorbic acid depended upon this ratio. 4 volumes of dialysate were equivalent to 1 volume of juice: the total volume of

reactants in each Warburg flask was 4.00 ml. and the standard volume of enzyme preparation used was 1.00 ml. Thus, if the reagents for one flask were made up from a particular dialysate, the rate of oxidation of the ascorbic acid would depend on the amount of Cu in 4.00 ml. of dialysate, i.e. on the amount of Cu from 1.00 ml. juice being dialysed. The rates of O_2 uptake for 1.00 ml. enzyme preparation and 4.00 ml. dialysate were thus directly comparable without further manipulation. Volume changes in the dialysing sacs were small enough to be neglected.

The Cu in the enzyme preparations was determined by first evaporating the juice in a platinum basin at 60–70° and incinerating the residue in a platinum crucible, great care being taken to prevent loss of material by spurting. The ash was then taken up in 2 × 2 ml. Cu-free glacial acetic acid with evaporation to dryness after each addition; the residue was taken up in water and made up to the original volume of the enzyme preparation. The amount of Cu was determined in these solutions both by the rate of oxidation of ascorbic acid and colorimetrically in matched 50 ml. Nessler tubes with the sodium diethyldithiocarbamate reagent. Nessler tubes were found to be more satisfactory than a colorimeter or photometer for the extremely small amounts of Cu being determined.

RESULTS AND DISCUSSION

The findings of Barron *et al.* [1935] already mentioned form a suitable starting point for the examination of the enzymic oxidation of ascorbic acid, since complete control of the reaction can be accomplished by arranging conditions to exclude all oxidation except that by an enzyme.

Some experiments were first performed with concentrations of $CuSO_4$ as catalyst ranging from 0.000001 *mM* to 0.001 *mM* in each flask with 25 μ -mol. ascorbic acid at pH 6.0. The reaction rates (Table 1) are in fair agreement with those observed by Barron *et al.*

Table 1. *Oxidation of ascorbic acid with $CuSO_4$ as catalyst*

pH = 6.0; temp., 25°; 25 μ -mol. ascorbic acid substrate.

<i>mM</i> $CuSO_4$	O_2 consumption μ l./hr.
0.000001	7
0.000005	39
0.00001	71
0.0001	221
0.001	709

In order to obtain preparations of cucumber oxidase which would be free from adventitious heavy metals, possible reaction intermediaries and other interfering bodies, the juice from cucumber tissue was dialysed for several days against Cu-free water until the dialysate was free from Cu. The activity of the juice decreased very slightly from day to day during dialysis, the original rate of O_2 uptake being about 1500 μ l. per hr. and the final rate after dialysis for 6–7 days being approximately 1000 μ l. per hr. Fig. 1 illustrates such an experiment. Catalytic oxidation by haemochromogens which possess, molecule for molecule, a fraction of the catalytic power of Cu salts, is excluded from these experiments since tests on the enzyme preparations for Fe with the dipyriddy reagent were either negative or so weakly positive that the amount of haemochromogen (assuming that all the Fe was present as such) would be insignificantly small. ✓

All salts having been removed from the enzyme preparations along with quinonoid and phenolic materials and organic molecules of small dimensions, some 50–100 ml. of the preparation were measured out for incineration and the resulting ash tested for Cu by measuring the rate of oxidation of ascorbic acid catalysed by 1.00 ml. of the solution of the ash. As the results in Fig. 2 show, the

Fig. 1.

Fig. 2.

activity of 1 ml. of dialysed enzyme preparation is much greater than that of 1 ml. of the solution of the enzyme ash: that is, the activity of a given amount of enzyme preparation is much greater than the catalytic activity of the amount of Cu in that enzyme preparation. The results of further experiments illustrating this principle are given in Table 2.

Table 2

Exp.	Cu found in juice $\mu\text{-mol./ml.}$	Ash activity $\mu\text{l. O}_2$ per hr.	Cu calculated from ash activity $\mu\text{-mol./ml.}$	Enzyme activity $\mu\text{l. O}_2$ per hr.	$\mu\text{l. O}_2$	$\mu\text{l. O}_2$
					hr. $\times \mu\text{-mol. Cu}$	min. $\times \mu\text{g. Cu}$
17	0.0020	16	0.0022	1036	470,909	123
18	0.0020	20	0.0030	990	471,430	126
19	0.0021	102*	0.0200	1000	500,000	131
20	0.0020	16	0.0022	1044	474,545	124

Cu was determined colorimetrically and also by calculation from the catalytic activity of the ash preparations using the values in Table 1 as a basis for the calculation. The last two columns express the activities of the preparations (1) as rate of O_2 uptake per hr. per $\mu\text{mol. CuSO}_4$ and (2) as rate of O_2 uptake per min. per $\mu\text{g. Cu}$.

* For 10 ml. juice.

Since, according to the results of Barron *et al.* [1935], no other metal than Cu is likely to be involved in the catalytic oxidation of ascorbic acid and since, under the conditions which obtained in these experiments, possible interfering substances were removed by dialysis, it is necessary to postulate some complex of Cu to account for the difference between the activity of the dialysed enzyme preparations and the activity of the corresponding amounts of ash solutions. That the oxidation is primarily concerned with the amount of Cu present in the preparations is shown by the reasonably concordant figures which express the activities of different samples of dialysed enzyme preparations as the rate of O_2 consumption per $\mu\text{g. Cu}$ (Table 2). The Cu complex postulated here is possibly

analogous to the Cu protein isolated by Kubowitz from potato. The dialysed enzyme preparations, however, clearly do not contain a polyphenol oxidase or a tyrosinase since experiments with *p*-cresol and catechol as substrate were negative, the rate of O_2 consumption being of the order of 5–10 μ l. per hr. (which is little more than the 'blank') compared with 1000 μ l. per hr. with ascorbic acid as substrate. Their behaviour and relatively constant copper: activity ratio suggest that there is present a discrete ascorbic acid oxidase.

Stotz *et al.* [1937] reported that the addition of cyanide and other substances forming insoluble complexes with Cu to cucumber juice inhibited the oxidase activity and that dialysis against water to remove the inhibitors restored the activity in every case except that in which cyanide had been added. This has been confirmed. In addition, experiments have been carried out with dialysed enzyme preparations to which NaCN and HCl were added to give a final concentration of *M*/100 in each case: the preparations with NaCN or HCl were dialysed separately against Cu-free water until the dialysates were free from cyanide or HCl. This required 2–3 days' dialysis, during which time the dialysates gave positive reactions when tested for Cu with sodium diethyldithiocarbamate. A further removal of Cu by dialysis against cyanide or HCl had therefore occurred, although Cu was no longer removable by simple dialysis against water. The enzyme preparations, after this further removal of Cu, were quite inactive in catalysing the oxidation of ascorbic acid.

It remained to restore the activity of the dialysed preparations. Samples of dialysed cucumber juice of known Cu content were dialysed with *M*/100 NaCN and *M*/100 HCl and then with Cu-free water until all cyanide or HCl had been removed (4–5 days). Three and ten times the calculated amounts of Cu were added as $CuSO_4$ to samples of the inactivated preparations and allowed to stand for a few hours at room temperature: the preparations were then dialysed against water for 24 hr. to remove any excess Cu.

Fig. 3. A, undialysed preparation (1 ml.). B, dialysed preparation (1 ml.). C, restored preparation (10 $\times$ excess Cu). D, restored preparation (3 $\times$ excess Cu). E, 0.00002 mM $CuSO_4$. F, 0.000006 mM $CuSO_4$. G, ash solution (1 ml.).

Table 3

	O_2 consumption μ l. per hr.
1 ml. dialysed preparation A	1248
1 ml. inactivated preparation B	0
1 ml. inactivated preparation + 0.00002 mM $CuSO_4$ C	834
1 ml. inactivated preparation + 0.000006 mM $CuSO_4$ D	558

Cu content of A. 0.0025 μ -mol./ml.

Cu content of A. 0.0022 μ -mol./ml. calculated from ash activity.

Cu content of C. 0.0030 μ -mol./ml.

The preparations inactivated with HCl remained inactive: this was probably due to the extensive denaturation and precipitation of protein which occurred during the inactivation period.

The preparations inactivated with NaCN were restored to activity and the results are shown in Table 3. The experiment is also recorded graphically in

Fig. 3, the curve for the O_2 uptake of the inactivated preparation being omitted as it was nil. The activity of the enzyme preparation was successfully restored to the extent of 67 and 45% by the addition of ten-fold and three-fold excesses of an inorganic Cu salt. Taken in conjunction with the inactivation of cucumber juice by the removal of Cu, with the relation between the oxidase activity and the Cu content of the dialysed preparations, and with the difference between the activities of Cu in the preparations and Cu in the ionic form, these facts provide evidence that there exists in autolysed cucumber juice a discrete ascorbic acid oxidase, and that the enzyme is a metal-protein in which the prosthetic group is Cu.

SUMMARY

The ascorbic acid oxidase activity of cucumber juice is proportional to the amount of Cu in the juice after all 'free' Cu and other extraneous materials have been removed by prolonged dialysis against water. This activity is much greater than the catalytic activity of ionic Cu or Cu in the form of a loose non-specific combination with protein.

Complete removal of Cu inactivates the cucumber juice.

Dialysed cucumber juice inactivated by further dialysis with NaCN has been restored to activity by the addition of excess inorganic Cu salt; the restored preparation was more active in the oxidation of ascorbic acid than the amount of Cu salt used for reactivation.

It is concluded that cucumber juice contains a discrete ascorbic acid oxidase, a metal protein complex in which the prosthetic group is Cu, and which is not identical with polyphenol oxidase.

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