

ON THE FUNCTION OF HEXURONIC ACID IN THE RESPIRATION OF THE CABBAGE LEAF

By ALBERT SZENT-GYÖRGYI

(From the Division of Chemistry, The Mayo Foundation, Rochester, Minnesota)

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It has been shown (3) in a previous paper (1928) that plants which contain a peroxidase system and the suprarenal cortex of animals contain a relatively high concentration of a substance called hexuronic acid. This substance is characterized by its high reducing power and its reversible oxidizability. It has been suggested that hexuronic acid is involved as a catalyst in the respiration of the cell. The subject of this paper is the inquiry into the part this substance plays in the respiration of the cabbage leaf.

If a part of the leaf is placed in the respirometer¹ in the presence of potassium hydroxide, it shows a vigorous oxygen uptake of between 2.5 and 6 c.mm. for each gm. of tissue each minute. This rate of oxygen uptake remains constant for more than an hour.

If, however, the leaves are ground in a meat mincer, which injures most of the cells and releases the intracellular fluid, and the pulp is placed in the respirometer, it shows a small oxygen uptake, between 1 and 2 c.mm. for each gm. each minute. This shows that mincing the leaves greatly injures the normal mechanism of respiration.

In experiments in the respirometer, the first 5 minutes are lost for observation because of the time necessary for the respirometer to reach equilibrium as well as the few minutes required to weigh the pulp and fill the respirometer. If at the beginning of the readings a sample is taken, it will be found to contain practically no reduced hexuronic acid. Since the acid is present in the intact leaf chiefly in the reduced condition, it is evident that the

¹ The thin marginal parts of inner leaves were used for these experiments.

reduced form disappears between the time of mincing and the beginning of the readings.

The disappearance of the reduced hexuronic acid can be followed by color reactions. 15 cc. of the Folin phenol reagent are placed in a series of beakers. The leaves are minced and the pulp quickly spread on a glass plate. At intervals of 2 minutes, samples of about 2.5 gm. of the pulp are placed in the reagent. After the first eight samples have been taken, the content of the beakers is filtered through muslin into test-tubes. The reduced hexuronic acid reduces the reagent without addition of alkali.

In the sample taken immediately after mincing, a deep blue color indicates the presence of a high concentration of reduced hexuronic acid. In the sample taken 2 minutes later, the color is moderately deep. In the third sample the color is rather faint. The fourth and fifth samples indicate only a trace; the remainder are negative.

This experiment shows that the relatively large amount of reduced hexuronic acid in the intact leaf disappears within the first 5 minutes after mincing. Since this disappearance does not take place if the pulp is kept in a vacuum, it is evident that the disappearance of the substance is due to its oxidation. During this interval, the pulp thus takes up oxygen at a rate of about the same order as that of the normal respiration. The absorption of oxygen lasts as long as reduced hexuronic acid is present. The pulp does not reduce oxidized hexuronic acid, or does so to a small extent only and indicates that the mechanism which brings about the reduction of hexuronic acid is damaged by the process of mincing to a greater extent than is the mechanism of the oxidation of the acid.

If the original concentration of reduced hexuronic acid is restored to the pulp by addition of this substance, a vigorous uptake of oxygen again takes place which lasts until the theoretic quantity of oxygen is taken up which is required for the reversible oxidation of the added acid. Fig. 1 shows the result of such an experiment.

Hexoxidase

If the pulp is quickly boiled, and then cooled, it will not be able to oxidize hexuronic acid. This shows that the oxidation of the acid in the fresh pulp is due to the presence of a thermola-

bile catalyst. For the sake of brevity this catalyst will be referred to as hexoxidase.

The first question to be studied was whether the hexoxidase is bound to the formed elements of the cell or is, in part at least, also present in the cell sap in solution. It was found that the hexoxidase is present to a large extent in the juice.

If the pulp is pressed through muslin, placed in the respirometer, and hexuronic acid is added, a great increase of oxidation shows the presence of the hexoxidase. The oxygen uptake, on

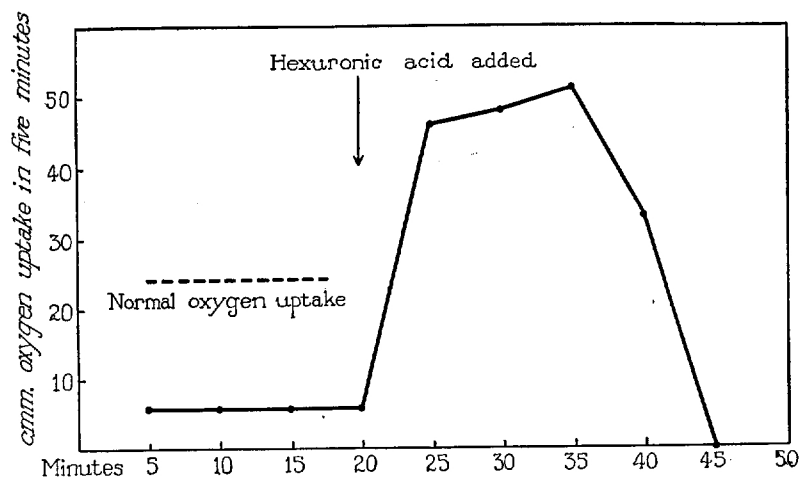


FIG. 1. The effect of hexuronic acid on the uptake of oxygen by minced cabbage leaves.

addition of the acid, will be the same if the juice is freed from all suspended material by centrifugation.

In Fig. 2 are shown the results of an experiment in which the pulp was pressed out at once and placed quickly in the respirometer.

If barium acetate is added to the juice a heavy precipitate is produced which can be separated in the centrifuge. The supernatant fluid shows undiminished activity.

If an equal quantity of saturated solution of ammonium sulfate is added to the juice, about half of the hexoxidase is precipitated.

If the juice is saturated with ammonium sulfate, all of the

hexoxidase will be found in the precipitate, which can be easily separated on the Buchner funnel. If the precipitate is dissolved in water or a phosphate buffer, it can be reprecipitated repeatedly practically without loss of activity. Such a preparation obtained by precipitation with ammonium sulfate does not show spontaneous uptake of oxygen in the respirometer.

If the ammonium sulfate precipitate is suspended in $m/15$ phosphate solution of pH 5.9 and is allowed to stand overnight in the ice box, an inactive precipitate will settle, which can be

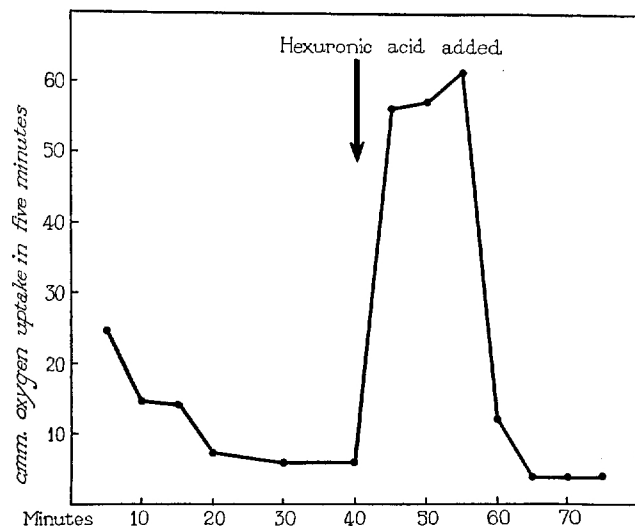


FIG. 2. The effect of hexuronic acid on the uptake of oxygen by juice pressed from cabbage leaves.

separated in the centrifuge, leaving a limpid, highly active enzyme solution.

All further observation will deal with the enzyme which had been precipitated from a solution saturated with ammonium sulfate and redissolved in $m/15$ phosphate buffer of pH 5.9 (corresponding to the pH of the cabbage juice).

The hexoxidase is resistant to many different chemical and physical agents. The preparations can be kept for approximately a week in the ice box without loss of activity. The wet ammonium

sulfate precipitate can be dried in the air or in a vacuum. The solution can be frozen in solid carbon dioxide. A solution of 1 per cent acetic acid, or of sodium carbonate or formalin, will not destroy its activity in 5 minutes. The dried preparation can be extracted with ethyl ether without loss of activity or by absolute alcohol or alcohol-ether with but little loss of activity.

Rapid heating to boiling will completely destroy the enzyme. Solutions of the enzyme or the wet ammonium sulfate precipitates are very sensitive to solvents like alcohol or acetone. Treatment with three times its own volume of methyl alcohol destroys 80 per cent of the activity. Acetone destroys it almost completely.

The enzyme is not sensitive to the cyanide ion, showing that in its action a mechanism usually classified as "oxygen activation" is not involved. Sodium cyanide 0.005 per cent has no effect on the rate of oxidation; 0.01 per cent has a small inhibitory action. High concentration, as 0.1 per cent, inhibits oxidation to a great extent. It has been shown by Dixon (1927) (1) and by me (1926) (2) that such a high concentration of cyanide inhibits enzymes in a non-specific way. Sharding's enzyme is inhibited by such high concentration, although oxygen activation is not involved in its action. As emphasized by Dixon (1927) (1) only the inhibition by small concentrations of cyanide is characteristic for oxygen activation.

As criterion for hydrogen activation the reduction of methylene blue is usually applied. It is easy to demonstrate that the reduction of methylene blue by hexuronic acid is not enhanced by the presence of hexoxidase. Also narcotics, such as 5 per cent of urethane or chloroform, have no inhibitory influence.

The insensitivity of the enzyme to cyanide gives further evidence of the significance of this enzyme, together with hexuronic acid, in the normal respiration of the cabbage leaf. It was found that the respiration of intact leaves is inhibited only to 6 per cent by the presence of 0.01 per cent of sodium cyanide. The respiration of thin slices of a plant containing a phenol-oxidase, the potato, was found to be inhibited in an identical experiment up to 60 or 75 per cent by the same concentration of cyanide.

The results show the mechanism of activation of hexoxidase to be different from that of all other oxidizing enzymes. So do the kinetics of its activity. If the rate of oxidation in varying con-

centrations of hexuronic acid is plotted, a straight line is obtained, which slopes but gently, and the rate of oxidation tends to become parallel to the abscissa in low concentrations of the acid. The results of two of the several experiments performed are shown in Fig. 3. The same result can also be obtained by a different method. If hexuronic acid is mixed with the hexoxidase and samples are taken at regular intervals and the quantity of the unoxidized acid is plotted against time, an almost straight line is

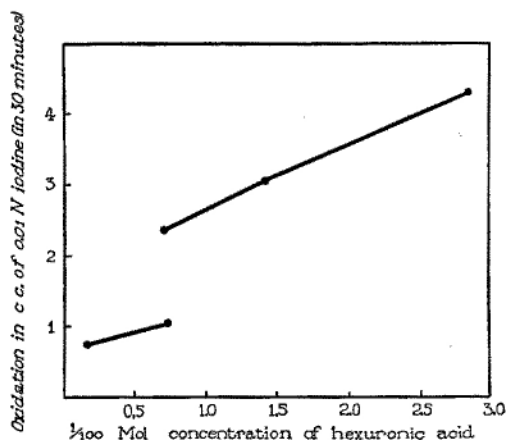


FIG. 3. The effect of concentration on the velocity of oxidation of hexuronic acid by hexoxidase.

obtained, until oxidation of the acid is almost complete. The experiments were carried out at room temperature in beakers with continuous mechanical shaking. The quantity of unoxidized hexuronic acid was followed iodometrically.

No other substance has been found besides hexuronic acid on which the hexoxidase will act. Pyrogallol, catechol, quinol *p*-phenylenediamine, the diamine plus naphthol, leucoindophenol, all remain unoxidized.²

² It should be mentioned that the pulp of cabbage leaves or the pressed juice oxidizes dibrom-indophenol white at a high rate. This reaction is sensitive to cyanide. By precipitation with ammonium sulfate the juice loses this activity.

Also glutathione³ remains unoxidized in the presence of hexoxidase. If, however, hexuronic acid is present, the glutathione is oxidized; the hexuronic acid plays the rôle of catalyst; it is oxidized by the enzyme and reduced by glutathione.⁴

A study of the velocity of oxidation of hexuronic acid shows that the oxidizing enzyme is of a complex nature. The probable mechanism by which the enzyme brings about its effect has been discussed in a recent publication (4). The curves which represent the velocity of oxidation of hexuronic acid in the presence of the enzyme support the hypothesis that an intermediate substance, x , is involved. If the rate of oxidation of hexuronic acid is rapid compared to the rate of oxidation of x , the line in Fig. 3 would be parallel to the abscissa. The gentle slope indicates that the rate of oxidation of x to the rate of oxidation of hexuronic acid by x stands as 1 to 3.

EXPERIMENTAL

*Experiment 1. Uptake of Oxygen by Minced Cabbage Leaves—*The outer leaves of the cabbage are rejected. The thick main ribs of the inner leaves are cut out with a sharp knife, and the leaves are quickly minced in a meat mincer, which reduces them to a relatively fine, juice pulp. A sample of 813 mg. is quickly weighed and transferred to a respirometer of the Warburg type and suspended there in 2 cc. of a M/15 phosphate buffer of pH 5.9.

The side arm of the respirometer is filled with 0.5 cc. of a solution of hexuronic acid, brought to pH 5.9 with disodium phosphate. This sample reduces 3.4 cc. of 0.01 N iodine and requires thus for its reversible oxidation about 200 c. mm. of oxygen.

0.4 cc. of potassium hydroxide solution is placed in the central tube of the respirometer to absorb carbon dioxide. The respirometer kept at 24° is closed and the readings which are begun after 5 minutes, are repeated at intervals of 5 minutes.

³ Crystalline glutathione was prepared by the method of Kendall.

⁴ Formaldehyde in the presence of the enzyme preparation leads to rapid disappearance of hexuronic acid (iodometrically) and of cysteine and glutathione. This is, however, not an oxidative process since it occurs anaerobically at the same rate. Acetaldehyde is only slightly active. Propionic aldehyde, heptylic aldehyde, crotonic aldehyde, benzaldehyde, cinnamic aldehyde, anisic aldehyde, vanillin, and dimethyl *p*-aminobenzaldehyde are inactive.

After four readings, the side arms are dumped. Since the side arms cannot be washed out, the dumping is not quantitative. The horizontal dotted line in the curve (Fig. 1) gives the oxygen uptake of the same weight of intact cabbage leaves.

Experiment 2. Uptake of Oxygen by Pressed Cabbage Leaves—Cabbage leaves are minced quickly. The pulp is wrapped in muslin, pressed by hand, and 2 cc. of the juice pipetted into the respirometer. Further details are the same as those given in Experiment 1. The curve obtained is shown in Fig. 2.

Experiment 3. Effect of Concentration on the Velocity of Oxidation—A 0.01 M solution of hexuronic acid is prepared by the addition of 8.9 mg. of hexuronic acid to a solution of hexoxidase in 5 cc. of phosphate buffer pH 5.9. The solution is gently shaken for 30 minutes and is then acidified with acetic acid and titrated with a solution of 0.01 N iodine. Similar experiments were made with solutions which contained 0.015 M, and with 0.03 M, concentrations of hexuronic acid. The influence of the concentration of hexuronic acid is shown in Fig. 3.

Preparation of Hexuronic Acid

The hexuronic acid used in the experiments was prepared in the chemical laboratory of The Mayo Foundation from suprarenal glands of beef. Since (for the purpose of constitutional analysis) greater amounts of hexuronic acid had to be prepared, the methods of preparation described elsewhere (1928) were applied for quantities of 25 kilos of glands. It was found, however, that the original method was inadequate for work on such a large scale. The original method was thus modified in several respects. By this modified procedure, I have obtained 4 gm. of crystalline hexuronic acid from 25 kilos of glands. The same yield has been obtained by my assistant, Miss Bair. More than 20 gm. of crystalline hexuronic acid have been prepared in this way.

The steps of the preparation were the following. The glands were cut from the freshly killed animals at the Swift packing plant at South St. Paul, Minnesota. They were at once trimmed, and packed in solid carbon dioxide, in which they were transferred to the clinic, where they were minced still in frozen condition. Then to every kilo of the pulp, 1 liter of 5 per cent trichloroacetic acid was added, and the whole mass was thoroughly mixed.

After half an hour, the fluid was separated in the Sharples centrifuge. 40 gm. of neutral lead acetate for each kilo of glands were dissolved in a small quantity of hot water. To every liter of the solutions of the glands 0.1 cc. of 20 per cent sodium cyanide was added. Two-thirds of the lead acetate solution was added to the solution; then a 10 N solution of sodium hydroxide was added with rapid agitation until the fluid distinctly turned the indicator brom-thymol blue. The rest of the lead acetate was then poured in. The mixture was cooled with ice for half an hour; the precipitate was separated in the Sharples centrifuge.

The precipitate was suspended in about 2 liters of water and 25 per cent sulfuric acid was added, until the fluid was distinctly acid to brom-thymol blue. Then the maximal amount of 20 per cent phosphotungstic acid was added which just did not leave any excess of this acid in the solution after being cooled with ice. (The necessary quantity was estimated on a small sample, sodium hydrosulfite being used as indicator for free phosphotungstic acid after the precipitate had been separated.) The solution was filtered on a suction filter.

The further steps of the method were similar to those described in the previous paper (1928).

SUMMARY

Evidence is given that hexuronic acid plays an important part in the respiration of the cabbage leaf. It connects, as hydrogen carrier, the system in which the molecular oxygen enters into reaction with the system which supplies hydrogen and is involved in the oxidation of the foodstuff. There is in the cabbage leaf a highly active enzyme which in the presence of oxygen rapidly oxidizes hexuronic acid to its reversible oxidation product. The name hexoxidase is used for this enzyme, the properties of which are described.

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