LXX. ASCORBIC ACID OXIDASE AND THE STATE OF ASCORBIC ACID IN VEGETABLE TISSUES

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Szent-Györgyi [1931] reported an enzyme in cabbage leaf which was capable of oxidizing vitamin C. Tillmans *et al.* [1932] found that the titration values of cucumber extract increased after treatment with hydrogen sulphide. Zilva [1934] made a similar observation on apple juice and found that the juice was capable of reversibly oxidizing the ascorbic acid of lemon juice. Ahmad [1935] reported that cut or shredded vegetables lost their vitamin C content in a short time. McHenry & Graham [1935] confirmed Ahmad's finding that minced vegetables lost their vitamin C, but showed that this could be restored by treatment with hydrogen sulphide. They concluded that vegetable pulp had no stabilizing mechanism for ascorbic acid. In their opinion, part of the increase noted on boiling certain vegetables was due to hydrolysis of ascorbic acid "esters".

Van Eekelen [1935], on the other hand, contended that the apparent increase of vitamin C on boiling potatoes is due to the inactivation of an enzyme which normally oxidizes ascorbic acid.

Tauber *et al.* [1935] isolated an enzyme from the pericarp of Hubbard squash and determined many of its properties. This enzyme has the property of immediately reversibly oxidizing ascorbic acid. The optimum temperature is 37° and the optimum *p*H 5·6. The enzyme showed different properties from Szent-Györgyi's cabbage enzyme.

Guha & Pal [1936] found that cabbage extracted with absolute alcohol and anhydrous sodium sulphate gave a higher value for vitamin C than by ordinary extraction. On extraction with ether and anhydrous sodium sulphate, no titration value was found. However, on heating the dry extract with water, a considerable vitamin C value was obtained. It was concluded that no enzyme takes part, but that the alcohol and ether extract an ascorbic acid ester, which is hydrolysed by heating with water.

Mack [1936] could not confirm Guha & Pal's results. He pointed out that absolute alcohol and ether would inactivate an enzyme and thereby prevent oxidation of the ascorbic acid. He showed that by extraction with sufficiently strong acid, or absolute alcohol, the true vitamin C value of the foodstuff is obtained. He found that the enzyme of cabbage was only partially inhibited even by *N* sulphuric acid. It was concluded that even though there may be a small increase on boiling due to the liberation of bound ascorbic acid, the effect was mostly due to the inactivation of the enzyme.

Levy [1936], testing the views advanced by McHenry & Graham, treated aqueous cauliflower extracts with both trichloroacetic acid and metaphosphoric acid and filtered. He then treated the filtrates with hydrogen sulphide and found that they showed far less ascorbic acid than those not treated with acid.

He then boiled aqueous cauliflower extracts and found that they suffered a large loss of ascorbic acid. He concluded that both the liberation of bound ascorbic acid and the inactivation of the enzyme take part in the apparent increase of ascorbic acid obtained on boiling these vegetables.

Srinivasan [1936] has isolated an oxidase from the expressed juice of drumstick pods (*Moringa pterygosperma*), which he considers to be specific for ascorbic acid.

The present investigation was divided into two parts:

A. An attempt to demonstrate either the presence or absence of an ascorbic acid oxidase in several fruits and vegetables.

B. A quantitative investigation of the activity of the enzyme found in cucumber.

A. Occurrence of ascorbic acid oxidase in fruits and vegetables

The fruits and vegetables listed below were treated as follows.

(1) A sample was weighed out and immediately ground with acid-washed sand and 10% metaphosphoric acid. The ascorbic acid was then determined by indophenol titration in the usual manner.

(2) A sample was ground in a mincing machine, the shredded pulp allowed to stand 5 min. and the juice expressed through muslin.

(*a*) The ascorbic acid content of the juice was determined as before.

(*b*) 5 ml. of the juice were treated with 5 ml. metaphosphoric acid (10%) to inactivate any enzyme, and then with hydrogen sulphide for 15 min. The flasks were stoppered and allowed to stand overnight. They were then treated with carbon dioxide until the escaping gas did not react with moistened lead acetate paper. The ascorbic acid content was then determined.

(*c*) 5 ml. of the expressed juice and 4 ml. of water were added to 1 ml. orange juice (*p*H 5·6) and the mixture heated for 10 min. at 37°. The ascorbic acid content was then determined to see if any enzymic reaction had taken place. Orange juice, as will be shown later, has no oxidase and suffers no loss of ascorbic acid on heating for 10 min. at *p*H 5·6 at 37°.

Results

Table I

		Ascorbic acid content			
No.	Fruit or vegetable	Meta-phosphoric acid extraction mg./g.	Juice mg./ml.	Juice after hydrogen sulphide mg./ml.	Action on orange juice*
1	Banana	0·06	0·01	0·08	+
2	Cabbage	0·60	0·01	0·22	+
3	Cantaloupe (Spanspek)	0·53	0·52	0·50	−
4	Carrots	0·24	0·04	0·23	+
5	Cucumber	0·10	0·005	0·13	+
6	Lettuce	0·22	0·26	0·25	−
7	Lucerne	2·20	2·30	2·25	−
8	Marrow (vegetable)	0·07	0·01	0·08	+
9	Onion	0·06	0·09	0·09	−
10	Peas (fresh green)	0·20	0·31	0·31	−
11	Potato	0·19	0·01	0·27	+
12	Spinach	0·75	0·79	0·76	−
13	String beans	0·19	0·02	0·16	+
14	Water melon	0·07	0·06	0·06	−

* + indicates ascorbic acid of orange juice oxidized. − indicates ascorbic acid of orange juice not oxidized.

It will be seen from Table I that bananas, cabbage, carrots, cucumber, vegetable marrow, potato, and string beans lose practically all their indophenol-reducing power on mincing, while the others, cantaloupe, lettuce, lucerne, onion, green peas, spinach, and water melon do not. The higher ascorbic acid contents of some of the juices were due to their being more concentrated than the whole vegetable in soluble constituents.

Those vegetables which lose their reducing power on mincing can also destroy the reducing power of orange juice, while the others cannot. All the juices which lose their reducing power can be brought back to normal by the hydrogen sulphide treatment outlined above. (Except cabbage, the enzyme of which, as noted by Mack, is not completely inhibited by dilute acids.)

B. The activity of cucumber juice

(1) Cucumber juice was mixed with orange juice [cf. Zilva, 1934] containing 0·40 mg. ascorbic acid per ml., in the proportions noted in Table II. The *p*H was 5·6, and the mixtures were heated at 37° for 10 min.

Table II. *The effect of cucumber juice on the ascorbic acid content of orange juice*

Conditions of experiment: *p*H, 5·6; temperature, 37°; time, 10 min., ascorbic acid content of orange juice = 0·40 mg./ml., final volume = 10 ml.

Volume of orange juice ml.	Volume of cucumber juice ml.	Ascorbic acid equivalent to 5 ml. cucumber juice mg.	Ascorbic acid remaining equivalent to 5 ml. cucumber juice mg.	Ascorbic acid oxidized %
0·0 (Negative control)	5·0	0	0	—
1·0	5·0	0·40	0	100
2·5	5·0	1·00	0	100
5·0	5·0	2·00	0·11	94·5
5·0	4·0	2·50	0·14	94·5
5·0	2·0	5·00	0·27	94·5
5·0	1·0	10·00	1·65	83·5
5·0	0·5	20·00	4·40	78·0
5·0 (Positive control)	0	*2·00	*2·00	0

* Actual amount of ascorbic acid present in the test solution.

(2) 5 ml. cucumber juice were mixed with 5 ml. ascorbic acid solution of the strength noted in Table III. Other conditions same as in Table II.

Table III. *The effect of cucumber juice on solutions of synthetic ascorbic acid*

Conditions of experiment: *p*H, 5·6; temperature, 37°; time, 10 min.; 5 ml. cucumber juice + 5 ml. ascorbic acid solution.

Ascorbic acid present mg.	Ascorbic acid remaining mg.	Ascorbic acid oxidized %
0·00 (Negative control)	0·00	—
0·16	0·00	100
0·31	0·00	100
0·62	0·00	100
1·25	0·00	100
2·50	0·00	100
5·00	0·22	95·4
10·00	3·74	62·6
10·00 No cucumber juice (Positive control)	7·26	27·4

It is shown that orange juice by itself does not lose any ascorbic acid by such treatment. The loss sustained by solutions of pure ascorbic acid when they are allowed to stand under unfavourable conditions has already been reported by Kellie & Zilva [1935]. The oxidation of ascorbic acid by cucumber juice is proportional to the amount of juice present.

That the ascorbic acid can be regenerated is shown by the following experiment:

A sample of cucumber juice was treated with metaphosphoric acid and hydrogen sulphide and found to contain 0·19 mg. per ml. of ascorbic acid. 2·5 ml. orange juice (*p*H 5·6, ascorbic acid 0·5 mg. per ml.) and 2·5 ml. water were added to 5 ml. cucumber juice, and the mixture heated for 10 min. at 37°. The reducing capacity disappeared. 5 ml. of this mixture were treated with 5 ml. 10% metaphosphoric acid and then with hydrogen sulphide in the usual manner. 1·1 mg. ascorbic acid were found in the solution. Of this, 0·47 mg. was from the cucumber juice, leaving 0·63 mg. from the 1·25 ml. orange juice present. This shows a recovery of 100%.

Summary

1. Several fruits and vegetables were investigated as to: (*a*) their ascorbic acid content by extraction with 10% metaphosphoric acid; (*b*) the ascorbic acid content of their expressed juices, before and after treatment with hydrogen sulphide; (*c*) the activity of the expressed juices in oxidizing the ascorbic acid of orange juice; all with a view to determining which, if any, contained an ascorbic acid oxidase.

2. Those vegetables which lost their indophenol-reducing power on mincing (banana, cabbage, carrots, cucumber, potato, string beans and vegetable marrow) were able to oxidize the ascorbic acid of orange juice, thus showing an ascorbic acid oxidase to be present.

3. Those which retained their ascorbic acid content (cantaloupe, green peas, lettuce, lucerne, onions, spinach and water melon) had no effect on orange juice, and hence have no oxidase.

4. The enzyme catalyses the reversible oxidation of ascorbic acid to dehydroascorbic acid, 100% recovery being obtained by hydrogen sulphide treatment.

5. The enzyme acts equally well upon natural ascorbic acid (in orange juice) and on synthetic ascorbic acid.

6. There is apparently no dehydroascorbic acid in the intact vegetable, this being formed only when a cut or crushed vegetable containing the enzyme is exposed to air.

7. There is no necessity to assume the presence of a stabilizing system for ascorbic acid, as proposed by McHenry & Graham. Those juices which retain their vitamin simply do not have any oxidase.

8. The findings of Mack that the oxidase of cabbage is only partially inhibited by dilute acid, and that if sufficiently strong acids are used during the extraction, the true vitamin C content of the foodstuff is obtained, are confirmed. If, however, the enzyme is given time to act before the acid is added, the vitamin will be oxidized.

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Note added 15 March 1937. After submission of this paper for publication the article of Kertesz, Dearborn and Mack [*J. biol. Chem.* **116**, 717] was received. These workers have found that some of the vegetables which I have reported to contain no oxidase apparently contain a small amount which acts after a longer period of time than was tried in my experiments. In order to see if this was true of South African varieties the following experiment (according to Kertesz) was carried out.

The vegetables mentioned in the table below were treated as follows.

(1) The ascorbic acid content after extraction with 10 % metaphosphoric acid was determined in the usual manner with indophenol.

(2) The vegetable was ground in a mincing machine and the juice expressed from the pulp. The ascorbic acid content of the juice was determined. One portion of the juice was heated 1 min. in a boiling water-bath to destroy any oxidase that may have been present. Another portion was left untouched. Both portions were then left for 3 hours at 37°. After this period the ascorbic acid contents of the two fractions were again determined. The results were as follows:

	Ascorbic acid		Heated		Unheated	
Vegetable	Acid extract mg./g.	Juice mg./ml.	Ascorbic acid remaining mg./ml.	% loss	Ascorbic acid remaining mg./ml.	% loss
Lettuce	0·27	0·28	0·07	75	0·12	57
Onions	0·05	0·07	0·07	0	0·07	0
Peas (fresh green)	0·35	0·55	0·51	7·3	0·55	0
Spinach	0·90	0·92	0·10	91	0·15	85

Since in no case is the value after heating higher than in the unheated juice, and in three cases actually lower, it is concluded that there was no oxidase present in the vegetables, even in small amounts. The enormous loss sustained by spinach is non-enzymic and was noted by Kertesz *et al.*, and was ascribed by them to its high iron and copper content. No doubt this is also true of lettuce. This loss in spinach is of great importance because the mode of preparation of this vegetable for consumption is very conducive to the destruction of its excellent vitamin C content. In order to minimize this loss, it should be served immediately after cooking. Kertesz has shown that this oxidation is irreversible.