XVI. COMBINED ASCORBIC ACID IN PLANT TISSUES

By ERIC JAMES REEDMAN AND EARLE WILLARD McHENRY

School of Hygiene, University of Toronto

(Received 29 November 1937)

SEVERAL chemical methods have been suggested for the estimation of ascorbic acid. Of these, titration with the dye dichlorophenolindophenol has met with most favour and has been extensively employed. Using this procedure, McHenry & Graham [1935] found that short periods of cooking caused an apparent increase in the titration values of certain vegetable tissues. A similar increase was obtained after acid hydrolysis of the tissues at room temperature. As an explanation of these increases McHenry & Graham postulated the presence in the foods of an ascorbic acid compound, insoluble in the extractants ordinarily used, and from which compound ascorbic acid was freed by hydrolysis.

Confirmation of these results was secured by Ahmad [1935], Guha & Pal [1936], and by Levy [1937]. Van Eekelen [1935] and Mack [1936] were unable to verify these results and both authors believed that the observed increase in titration values was caused by the destruction of "ascorbic acid oxidase". This explanation assumes that an extract of fresh tissue contains reduced ascorbic acid and the oxidase system, the latter of which converts some of the ascorbic acid into dehydroascorbic acid. If the tissue is heated prior to extraction the enzyme is destroyed, no oxidation of ascorbic acid can occur, and the titration value will be higher. Since McHenry & Graham had estimated both reduced ascorbic acid and dehydroascorbic acid in both fresh and heated tissues, this explanation is not applicable to their results. Mack & Tressler [1937] reported the production of reducing substances by heating dehydroascorbic acid in almost neutral solutions and believed these to be physiologically inactive degradation products. They explained the apparent increase of free ascorbic acid after heating to be due to the formation of such products. However, the ascorbic acid liberated from the protein complex is physiologically active, as will be shown later in this paper.

While chemical evidence had indicated that a substance similar to ascorbic acid was released by hydrolysis from certain tissues, the identity of the substance had not been established. In this communication chemical, spectroscopic and biological data are reported, indicating that the substance secured by hydrolysis of the protein complex is ascorbic acid.

CHEMICAL STUDIES

The protein-ascorbic acid compound is soluble in cold water and may be precipitated from a water extract of plant tissue by the addition of trichloroacetic acid or metaphosphoric acid. This is a convenient method for separating combined ascorbic acid from the reduced and reversibly oxidized forms. The precipitate, containing protein and ascorbic acid in combination with protein, is hydrolysed with 0.2-1.0% hydrochloric acid. Subsequent precipitation with the same protein precipitants removes protein, leaving the substance which reduces

phenolindophenol in solution. Such a solution behaves similarly to one of pure *l*-ascorbic acid and gives negative tests for other reducing substances. "Ascorbic acid oxidase" destroys the reducing properties of the solution as does oxidation by chemical reagents. Qualitative and quantitative analyses show that the precipitated material, formerly combined with ascorbic acid, is protein.

Table I summarizes a series of heat treatments of potato tissue, and Table II gives the amounts of ascorbic acid obtained by various extractions of similar

Table I. *Effect of heating treatments on the free ascorbic acid content of potato tissue*

Treatment	Total free ascorbic acid mg. per 100 g.
1. Raw—extraction with $\text{CCl}_3\text{COOH}-(\text{HPO}_3)_4$	14.1
2. Heated 3 min. at 98° under CO_2	19.5
3. Heated 15 min. at 98° without CO_2	11.2
4. Heated 30 min. at 98° without CO_2	6.9
5. Heated 30 min. at 98° with 0.5% citric acid	10.0
6. Heated 30 min. at 98° with NaHCO_3 to pH 9–11	1.3
7. Heated as above with oxygenation	Nil
8. Dehydrated at $35-40^\circ$	5.8

Table II. *Effect of various extraction treatments on the titration values for ascorbic acid*

Treatment	Total ascorbic acid mg.
1. 25 g. potato tissue extracted with cold water	7.4
2. 25 g. potato tissue extracted by heating 3 min. at 98° under CO_2 in distilled water	7.4
3. 25 g. potato tissue extracted with CCl_3COOH after hydrolysis with 1% HCl	7.5
4. 25 g. potato tissue extracted with cold $\text{CCl}_3\text{COOH}-(\text{HPO}_3)_4$	5.4
5. (a) 25 g. potato tissue extracted with cold water	7.2
(b) 20% CCl_3COOH added to extract and precipitate removed	5.3
(c) Centrifuged precipitate hydrolysed with dilute acid	1.8
6. 25 g. potato tissue extracted with cold absolute alcohol	3.5
7. 25 g. potato tissue extracted with absolute alcohol after heating	7.1

tissue. All determinations were made by titration with 2:6-dichlorophenol-indophenol. Samples of tissue were taken from a well mixed, evenly sliced lot to prevent variation in the original ascorbic acid content. As found by Guha & Pal [1936] the heating of an absolute alcohol extract of potatoes causes an increase in titration value. Since "ascorbic acid oxidase" is inactivated by absolute alcohol, and is inhibited by the mixture of trichloroacetic and metaphosphoric acids used in extraction, the increase cannot be due to destruction of the oxidase. Other results in Table II tend to confirm the presence of protein-ascorbic acid.

In Table III the effects of heating various plant tissues on the values found for ascorbic acid after extraction and titration are given. Some tissues show an appreciable increase, others only a decrease after similar treatment. Fruits give a more gradual decrease after heating and in no case an increase. Apparently protein-ascorbic acid occurs only in some vegetable tissues.

Table IV records the amounts of ascorbic acid, dehydroascorbic acid and protein-ascorbic acid found in some varieties of potatoes. Ascorbic acid was estimated by titration of a trichloroacetic-metaphosphoric acid extract; dehydroascorbic acid was determined after treatment of the extract with hydrogen sulphide; and protein-ascorbic acid after preliminary hydrolysis with 1% hydrochloric acid as outlined later.

Table III. *Effect of controlled heating on the titration values of plant tissues*

Tissue	Total ascorbic acid (mg. per 100 g.)	
	Raw	After heating
A. Increase on heating:		
Cauliflower	19.0	31.0
Hubbard squash	3.1	4.1
Beet	2.7	6.2
String bean	1.4	2.1
Carrot	1.2	2.6
Parsnip	3.7	6.1
Red clover	26.2	42.3
B. Decrease on heating:		
Spinach	18.0	13.0
Cabbage	15.0	12.0
Onion	8.9	3.1
Broccoli	32.0	22.0
Corn	7.6	5.1
Peas	14.0	8.1
Asparagus	12.0	8.2
Turnip	35.0	18.0

Table IV. *Ascorbic acid content of some varieties of potatoes*

Variety	Ascorbic acid (mg. per 100 g.)			
	Reduced	Dehydro	Combined	Total
A. After storage for 5 months—1935:				
Katahdin	9.2	3.5	5.0	17.7
Early Ohio	6.1	6.9	7.5	20.5
Green Mountain	7.3	6.5	7.1	20.9
Dooley	6.7	2.0	10.7	19.4
Irish Cobbler	9.3	2.3	9.1	20.7
Carmen No. 1	5.2	8.7	5.6	19.5
B. After storage for 6 weeks—1936:				
Evergreen	13.4	3.3	5.4	22.1
Eureka	16.2	3.1	3.7	23.0
Delaware	13.8	4.8	5.2	23.8
Netted Gem	13.7	4.3	3.3	21.3
Irish Cobbler	15.7	1.2	14.5	31.4

Fig. 1. Hydrolysis curve of liberation of combined ascorbic acid in 0.2 % HCl.

No clear proof has been obtained as to whether protein-ascorbic acid is a chemical compound or an adsorption complex. Fig. 1 shows a hydrolysis curve obtained by hydrolysing aliquot samples of potatoes with 0.2% hydrochloric acid for various intervals. Fresh, whole potato tissue was chopped and carefully mixed. 25 g. aliquots were immediately ground under 50 ml. 0.2% hydrochloric acid, the mixture placed in flasks filled with carbon dioxide and then tightly stoppered. The flasks were placed in the dark at 40° F. as soon as possible. The hydrolysis interval included the time taken in grinding the sample under acid. After suitable periods total ascorbic acid was determined, combined ascorbic acid being the difference between the total value after hydrolysis and that before hydrolysis found in trichloroacetic-metaphosphoric acid extracts. Since the amount of ascorbic acid liberated is logarithmically correlated with time of hydrolysis until a maximum is reached, it seems likely that actual combination does exist between protein and ascorbic acid in the original tissue.

SPECTROPHOTOMETRIC EXAMINATION

For these estimations the Hilger medium quartz spectrograph was used with an iron arc as the source of light. The exposure time was 2.5 min. in all cases. In preparing the solution of combined ascorbic acid for analysis it was found necessary to use hydrochloric acid and trichloroacetic acid in the proportions of 100 to 20. This solution was first tested alone for absorption and was found not to interfere in the region desired (Pl. II A, 1, 7; Pl. II B, 1, 6). It was then used as solvent for all determinations. The pH of all solutions was kept constant since this is an important factor in spectrographic estimations.

Pl. II A shows the absorption spectra of solutions of synthetic *l*-ascorbic acid of various concentrations. The position of the absorption edge was found to shift toward the longer wave-lengths with increasing concentration, the edge being at 2813 Å. for a concentration of 100 mg. per 100 ml. solution. Spectra 2, 3, 4, 5, 6, Pl. II A, are for ascorbic acid solutions of 100, 50, 25, 12 and 6 mg. per 100 ml. respectively. No. 8 is the spectrum of the iron arc alone. The number of absorption centres is proportional to the concentration ($\alpha = KC$). The maximum absorption is found at 2650–2651 Å. Dilute solutions show this band only, which is specific for reduced ascorbic acid.

Pl. II B compares the spectrum of synthetic *l*-ascorbic acid (2) with that of ascorbic acid liberated from the protein complex (4). Qualitative estimations only were made. The solution for test was made by hydrolysing a precipitate of combined ascorbic acid (precipitated from a water extract with trichloroacetic acid) with 1% hydrochloric acid, and further precipitating with trichloroacetic acid. The hydrolysed precipitate yields reduced ascorbic acid which is not precipitated by the further addition of trichloroacetic acid. By this means clear colourless solutions were obtained for analysis containing only that ascorbic acid formerly combined in the tissues and now in reduced form. Solutions thus prepared and solutions of *l*-ascorbic acid give characteristic absorption spectrophotometrically (2, 4). This absorption is removed in both cases by oxidation of the ascorbic acid present, the spectra of these solutions then being as for the solvent alone (3, 5). This indicates that the solutions contained reduced ascorbic acid, hydrochloric acid and trichloroacetic acid only, and confirms the chemical evidence that reduced ascorbic acid is hydrolysed from the protein precipitate.

PLATE II A

PLATE II B

THE BIOLOGICAL ASSAY

It has frequently been observed that young guinea-pigs maintained on a diet deficient only in vitamin C rapidly lose weight and develop marked scorbutic symptoms in 21–28 days. Many die within this time, while very few survive a 4-week period on a basal scorbutic diet or one extremely low in vitamin C. Coward & Kassner [1936] used the above observations as a basis for the quantitative assay of vitamin C, and have obtained a graduation of increase in weight in proportion to the amount of antiscorbutic material received. This method, based on the weight increase of young guinea-pigs, combined with autopsy findings and tissue analyses, was used to test the antiscorbutic properties of the protein-ascorbic acid compound.

For this purpose potato tissue was found to be a convenient source, and a precipitate consisting of protein and ascorbic acid in combination with protein was prepared from a water extract of this tissue. 2500 g. of carefully washed new potato tissue were ground into and extracted with 1 l. of cold, carbon dioxide-saturated, distilled water for 5 min. The material was then pressed through a linen filter, the liquid being centrifuged to remove suspended starch. The protein-ascorbic acid compound was precipitated from solution by means of glacial metaphosphoric acid. This precipitate was separated by centrifuging and was found to consist of protein and reduced ascorbic acid in combination with protein. A fresh quantity of precipitate was prepared daily, suspended in water and fed immediately to the test animals by means of dropping tubes. The average moisture-free weight of precipitate obtained was 10.2 g., containing the equivalent of 30 mg. of reduced ascorbic acid as found by indophenol titration after hydrolysis.

Male guinea-pigs weighing 250–300 g. were used as test animals. These animals had been on a normal diet previous to the experiment. Three groups of animals were used in the first study, receiving synthetic *l*-ascorbic acid, combined ascorbic acid and the basal diet only, respectively. The basal diet as used in this laboratory was as follows:

Bran	...	...	...	...	45 %
Crushed oats	...	...	...	...	25 %
Powdered skimmed milk	...	...	...	...	25 % ¹
Yeast	...	...	...	...	3 %
CaCO ₃	...	...	...	...	1 %
NaCl	...	...	...	...	1 %

The animals received a fresh weighed portion of this diet daily, the same amount of food being placed before each animal. The animals were maintained in separate cages and were weighed daily. Synthetic *l*-ascorbic acid was dissolved in water to a concentration of 2 mg. per ml., and 1 ml. of a freshly prepared solution fed daily to each of 10 animals in the first group. The second group, consisting of 15 animals, received combined ascorbic acid in amounts sufficient to approximate 2 mg. of reduced ascorbic acid. The third group of 20 animals received only the basal diet. All animals were given 1 ml. of cod liver oil twice a week. The supplementary feedings were made orally by means of dropping tubes.

Table V gives a summary of the weight increases of these animals. Fig. 2 represents growth response on the basal scorbutic diet only, Fig. 3 on a diet supplemented by oral administration of 2 mg. of synthetic *l*-ascorbic acid daily and Fig. 4 on the same diet supplemented with the precipitate containing

¹ Heated in open trays under oxygen.

Table V. *Weight increases of animal groups*

Group	Supplement	Average weight of group (g.) at end of each week							Average increase in weight
		0	1	2	3	4	5	6	
I	2 mg. <i>L</i> -ascorbic acid	252.6	241.6	245.4	278.2	287.2	292.6	306.0	+54.4
II	Protein-ascorbic acid precipitate	278.4	272.8	279.6	269.6	265.2	285.0	310.8	+32.4
III	Basal diet only	234.7	222.4	219.2 (6 died)	206.9 (5 died)	188.0 (3 died)	— (1 died)	—	—

combined ascorbic acid. The curves given are representative of a larger number of animals used. The weight increases obtained with synthetic and combined ascorbic acid were not strictly comparable, possibly because of the difficulty in maintaining the level of combined ascorbic acid at 2 mg. per day. It was impossible to check chemically each feeding and it seems likely that the group on combined ascorbic acid did not receive exactly this amount, and that the precipitate varied somewhat in the amount of combined ascorbic acid contained.

All the animals receiving the basal scorbutic diet only (Group III) died within the period of the experiment. Outward symptoms of scurvy were pronounced at the end of the third week, with partial paralysis of the hind legs, ruffled hair, and swelling and signs of haemorrhage at the knee joints. Autopsy revealed marked haemorrhage, with swollen costochondral rib junctions. The animals in Groups I and II showed no outward symptoms of scurvy, and at autopsy no sign of haemorrhage was revealed in either group. Adrenal ascorbic acid analysis has been found a valuable key to the state of vitamin C nutrition in animals. Analysis of representative tissues from each group of animals resulted in low values for the animals of Group III, while the ascorbic acid content of the adrenal glands of Groups I and II was normal for this tissue.

In a further survey two groups of animals were placed on scorbutic diets. The first group of 5 guinea-pigs was allowed to remain on this diet for a period of 3 weeks, at which time freshly prepared combined ascorbic acid was given daily for an additional 3 weeks. The animals in this group had an average initial weight of 391.0 g., which fell to 343.4 g. at the end of the 3-week period, at which time the animals showed outward symptoms of scurvy. One animal died on the 25th day, autopsy revealing haemorrhages. During the second 3-week period on combined ascorbic acid the outward symptoms of scurvy disappeared, and no scorbutic symptoms could be found on autopsy.

The second group of 10 animals in this experiment received the basal diet supplemented with the protein-ascorbic acid compound which was hydrolysed and then subjected to oxidation processes specific for reduced ascorbic acid. In a period of 4 weeks frank scurvy was evident. Autopsy revealed haemorrhages typical of scurvy, the condition of the animals appearing similar to that of animals on a basal scorbutic diet only.

These experiments definitely indicate that reduced ascorbic acid in a combined form with protein was the effective agent in preventing and curing scurvy in these experimental animals. It was satisfactorily demonstrated that animals can utilize ascorbic acid in combination with protein as antiscorbutic. Since ascorbic acid is hydrolysed from the protein-ascorbic acid compound by hydrochloric acid as dilute as 0.2% it seems reasonable to conclude that hydrolysis takes place in the digestive tract to yield reduced ascorbic acid which is utilized as such.

Fig. 2. Weight chart of animals receiving the basal scorbutic diet only.
(D.S. = died of scurvy.)

Fig. 3. Basal diet supplemented with 2 mg. synthetic L-ascorbic acid daily.
(K.N. = killed normal.)

Fig. 4. Basal diet supplemented with protein-ascorbic acid compound.
(K.N. = killed normal.)

DISCUSSION

The finding that vitamin C occurs in a protein-ascorbic acid complex as well as in the form of reduced and reversibly oxidized-ascorbic acid is of importance in regard to the wide distribution and relatively high concentration of this factor in plant tissues. A number of protein complexes occur naturally, and there is evidence that other vitamins may exist in other than free form. Lovern *et al.* [1937] report that the occurrence of vitamin A is not confined to oil solutions, while Schultz [1936] states that the water-soluble factor H occurs only in a combined form with protein, and is liberated by hydrolysis. Physiologically such compounds would be more slowly absorbed in the animal body, and possibly more stable than the free vitamin.

Since protein-ascorbic acid is insoluble in trichloroacetic or metaphosphoric acid, suitable modifications should be introduced in chemical procedures using these acids for extraction. These involve preliminary hydrolysis of a weighed sample of tissue for 1 hr. with 1 % hydrochloric acid. Suitable precautions should be taken to prevent atmospheric oxidation. Reduced ascorbic acid and dehydro-ascorbic acid are then determined in the hydrolytic extract, the residue after hydrolysis being further extracted with trichloroacetic-metaphosphoric acid and the total ascorbic acid determined in the usual manner. The total ascorbic acid present in the tissue may be calculated from the sum of the separate titration values of the two extracts. A simple trichloroacetic-metaphosphoric acid extraction of the tissues determines reduced ascorbic acid only. Dehydro-ascorbic acid may be estimated by treatment with hydrogen sulphide. This method has given satisfactory results in this laboratory and was used in the analyses reported.

SUMMARY

1. Proof of the presence of a combined form of ascorbic acid (vitamin C) in certain plant tissues has been obtained through chemical, spectrographic and biological assay. This form occurs as ascorbic acid in combination with protein, the protein-ascorbic acid compound readily yielding reduced ascorbic acid and protein on hydrolysis. Ascorbic acid in combined form has been shown to be equivalent to reduced ascorbic acid in antiscorbutic properties.

2. Combined ascorbic acid has not been found to occur in other than certain vegetable tissues. In some of these it constitutes a fairly large percentage of the total ascorbic acid present, but is not estimated by the usual chemical method of analysis since it is insoluble in the extractants used.

3. Suitable procedures are given for the estimation of combined ascorbic acid in those tissues containing this form. The results of some analyses using this method are recorded.

The authors wish to acknowledge the interest of Dr C. H. Best in this work. Spectrographic estimations were made with the aid of Dr Bateson of the MacLennan Laboratories, University of Toronto. This research was facilitated by a grant from the Banting Research Foundation, Toronto, to one of the authors (E. J. R.).

REFERENCES

- Ahmad (1935). *Biochem. J.* **29**, 275.
Coward & Kassner (1936). *Biochem. J.* **30**, 1719.
Guha & Pal (1936). *Nature, Lond.*, **137**, 946.
Levy (1937). *Nature, Lond.*, **138**, 933.
Lovern, Edisbury & Morton (1937). *Nature, Lond.*, **140**, 276.
McHenry & Graham (1935). *Nature, Lond.*, **135**, 871.
—— (1935). *Biochem. J.* **29**, 2013.
Mack (1936). *Nature, Lond.*, **138**, 505.
—— & Tressler (1937). *J. biol. Chem.* **118**, 735.
Schultz (1936). *Med. u. Chem. Abhandl. med.-chem. Forschungsstatten I. G. Farbenind.* **3**, 143.
Van Eekelen (1935). *Nature, Lond.*, **136**, 144.