Effect of Mastication on the Ascorbic Acid Content of Raw Vegetables

By G. N. JENKINS, *Physiology Department, St Bartholomew's Medical College, London*

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It has been stated by Pyke (1944) that during the mastication of raw vegetables significant losses of ascorbic acid may occur owing to the action of ascorbic acid oxidase liberated from the damaged cells. As it has not been proved that plant enzymes are responsible for this alleged loss it was felt desirable to investigate the matter further.

EXPERIMENTAL

Proof that apparent loss is not due to ascorbic acid oxidase

The effect of mastication was investigated by chewing, without swallowing, a weighed quantity of a sample of raw, well-mixed shredded cabbage for 1 min., after which the contents of the mouth were ejected and stirred with a 20% solution of metaphosphoric acid. The mouth was washed out with 10 ml. of water and the washings were added to the acid. The ascorbic acid content of the unchewed and of the chewed vegetable was then estimated with 2:6-dichlorophenol-indophenol in the usual way (Harris & Olliver, 1942). This experiment regularly showed a considerable apparent loss of ascorbic acid. To determine whether this loss was caused by ascorbic acid oxidase the experiment was repeated on samples of cooked cabbage in which, of course, no enzyme was active, and the loss was always at least as great as with raw cabbage, proving conclusively that no plant enzyme is involved.

This finding is illustrated by the experiment, typical of many, recorded in Table 1.

Table 1. *Effect of mastication on apparent ascorbic acid content of raw and cooked cabbage*

	Ascorbic acid found in 20 g. of cabbage (mg.)	Ascorbic acid found in 20 g. of cabbage after chewing (mg.)	Loss (%)
Raw: 1st sample	13.5	5	63
Duplicate	13.5	5	63
Cooked: 1st sample	6	2	67
Duplicate	6	1	83

Although in the above experiment the effect of chewing is striking both for raw and cooked cabbage, the samples are not strictly comparable as their original ascorbic acid content differed considerably.

In later experiments the weights of raw and cooked cabbage were chosen to provide equal amounts of ascorbic acid and the result was again precisely the same, viz. loss from cooked cabbage was at least equal to, and usually slightly greater than, that from the raw vegetable.

As a further check, the rate of oxidation *in vitro* of ascorbic acid by ascorbic acid oxidase in ground raw vegetables was measured.

Several samples, each of 5 g., of thoroughly mixed shredded cabbage leaves were weighed out and the ascorbic acid content of three of them determined. Other comparable samples were thoroughly ground up with sand and a little water for either 90 sec. or 5 min., a procedure which resulted in much more severe damage to the cells than occurs in mastication, since during the latter process the vegetable is broken into fine but relatively intact pieces. To the ground material 20% metaphosphoric acid was added to inhibit enzyme action and the remaining ascorbic acid was then estimated.

Table 2. *Effect of grinding raw cabbage on its ascorbic acid content*

	Ascorbic acid before grinding (mg./100 g.)	Ascorbic acid after grinding (mg./100 g.)	
		90 sec. grinding	5 min. grinding
	155	157	44
	164	132	—
	169	170	—
	—	140	—
Mean	163	150	—
% loss		8	73

The results (Table 2) show that the loss during 90 sec. grinding is small, and provide further support for the conclusion that ascorbic oxidase is not an important cause of the apparent loss of ascorbic acid during mastication of raw vegetables.

The two previous experiments have been repeated on two vegetables more frequently eaten raw than cabbage, namely, watercress and parsley. In both cases, the losses from both raw and cooked samples have been similar, with a tendency for a greater loss in the cooked. The losses during grinding of these vegetables with a pestle and mortar for 1 and 2 min. have also been so small as to confirm the view that ascorbic oxidase does not significantly influence the ascorbic acid content of vegetables during mastication (Table 3).

Table 3. *Effect of mastication on apparent ascorbic acid content of raw and cooked parsley and watercress*

	Watercress*: Ascorbic acid		Parsley*: Ascorbic acid	
	mg./100 g.	mg. in portion chewed	mg./100 g.	mg. in portion chewed
Raw, unchewed	140 } 138.5 137 }	2.8	294 } 232 } 270 282 }	2.7
Raw, chewed	57 } 75 } 70 75 }	1.4	132 } 219 } 173 180 }	1.7
<i>Apparent loss</i>		1.4 (50%)		1.0 (37%)
Cooked, unchewed	74 } 68 63 }	2.7	103 } 77 } 90	2.2
Cooked, chewed	36 } 20 }	1.4† 0.8†	51 } 55 } 53	1.3
<i>Apparent loss</i>		1.3 (48%)† 1.9 (70%)† (Mean 1.6 (59%))		0.9 (41%)
Loss of ascorbic acid after grinding: for 1 min.		Watercress	Parsley	
for 2 min.		5%	13%	
		18%	—	

* In an attempt to reduce sampling errors, leaves and umbels only were taken.

† Quoted separately owing to divergence.

Table 4. *Effect of mastication on 10 g. of cooked cabbage followed by treatment of extract with H₂S*

	Ascorbic acid before chewing (mg.)	Ascorbic acid in extract after chewing (mg.)	Ascorbic acid remaining (%)	Ascorbic acid in extract after H ₂ S (mg.)	Ascorbic acid which formed dehydro-ascorbic acid (% of initial)	Not recovered (%)
1st exp.	2.5	0.70	28	1.83	44	28
Duplicate	2.5	0.25	10	1.60	52	38

Other possible causes of loss

With ascorbic acid oxidase thus eliminated as the cause of this apparent loss there would appear to be at least four other possible explanations. They are:

(1) Swallowing or drainage down the oesophagus of juice expressed from the vegetable.

(2) Oxidation of ascorbic acid by substances present either in saliva or vegetables.

(3) Presence of substances in saliva which interfere with the estimation of ascorbic acid.

(4) Absorption of ascorbic acid from the mouth.

Each of these possibilities was investigated in turn and from what follows it appears that (2) is the most important.

Unimportance of drainage into oesophagus. An experiment to determine whether any of the contents of the mouth drained down the oesophagus during mastication with voluntary inhibition of swallowing was kindly carried out by Prof. H. A. Harris. The subject chewed cabbage mixed with barium meal and was observed by X-rays during the 1 min. mastication. No trace of barium meal was observed in the oesophagus either visually or photographically until swallowing was carried out voluntarily at the end of the minute. Hence, it seems unlikely that the loss of ascorbic acid down the oesophagus would explain the effect.

The slow reversible oxidation of ascorbic acid by saliva on standing in acid extracts. To investigate whether saliva contains any substances which oxidize ascorbic acid, or interfere with the reaction between ascorbic acid and the dye, 3 ml. of saliva were taken and added to 0.5 mg. of ascorbic acid, dissolved in 10 ml. of 5% metaphosphoric acid, and after standing for 30 min. the mixture was titrated. The reading indicated that the solution then contained less than 0.02 mg., that is, a loss of more than 96% had occurred. As this result was later reproduced with boiled, as well as unboiled, saliva it proved that a non-enzymic substance had either oxidized the ascorbic acid or else was reoxidizing the dye almost as quickly as it was reduced by the ascorbic acid during the titration.

Subsequent experiments have shown that both of these reactions may occur but that the relatively slow oxidation of ascorbic acid (mainly to dehydroascorbic acid) in the acidified extract is the more important in the mastication experiments. In fact, if the titration is carried out rapidly, reoxidation of dye does not affect the titre appreciably as will be shown later. The fact that oxidation by saliva of ascorbic acid to dehydroascorbic acid had taken place in the acidified extract was proved by treating part of a chewed vegetable extract with H₂S at pH 3.5, a process which reduced the dehydro compound to ascorbic acid (Harris & Olliver (1942); this was found to result in a considerable regeneration of the original ascorbic acid content (Table 4). Complete recovery of the ascorbic acid

was never obtained in these experiments, however, a point which will be discussed later.

The relatively small effect of saliva during the titration

The effect of saliva on the reoxidation of reduced 2:6-dichlorophenol indophenol was demonstrated by decolorizing some dye exactly by means of ascorbic acid in metaphosphoric acid solution and then adding saliva, after which the pink colour of the dye slowly returned and its intensity increased during the following few minutes. The effect was not specific for the reduction of the dye by ascorbic acid; a dye reduced by other substances was just as readily re-oxidized by saliva. There was found to be a great variation in the potency of saliva from different individuals and from the same individual at different times. The saliva of some subjects was occasionally completely negative.

When titrating the acidified extracts of chewed vegetables it was observed that after an end-point had been obtained the pink colour of the dye slowly returned after a few minutes' standing, a fact which often caused difficulty in judging the end-point accurately. The results were more uniform when a standard time, usually 15 sec., was allowed for the complete titration. This reoxidation was presumably due to the oxidizing substance in saliva. The relative unimportance, however, of this reaction in the mastication experiments was demonstrated as follows: 1 ml. of saliva was added to 10 ml. of ascorbic acid solution in metaphosphoric acid, and allowed to stand 30 min., and was then titrated rapidly. In one experiment, the volume of solution required to reduce a fixed amount of dye was 1.3 ml., and this volume contained, therefore, 0.12 ml. of saliva. When this volume of saliva was added to dye which had been exactly decolorized with ascorbic acid, the red colour became just perceptible within 1 min. and was unlikely, therefore, to have brought about significant reoxidation of the dye during the much shorter time occupied by an actual titration. The conclusion is, therefore, that reoxidation of dye is a much less important contributory factor than is the slow oxidation of ascorbic acid in the acidified extract; further evidence provided later on this point shows that reoxidation of dye during titration may be neglected provided the titration is carried out rapidly.

The identity of the oxidizing agent in saliva

Of the known constituents of saliva which have been tested, nitrite is the only substance which has been found to bring about the two observed effects, namely, slow oxidation of acid solutions of ascorbic acid and reoxidation of dye during titration. Several inactive substances contained in saliva, however, enhance the oxidizing effect of nitrite. For example, both potassium thiocyanate and creatinine, when added with nitrite, caused a return of the colour of reduced dye to a greater intensity than with nitrite alone, though neither substance without nitrite had any effect. The activity of any particular sample of saliva is thus probably primarily due to its nitrite content but is influenced by other substances. The correlation between the nitrite content of a saliva, as determined by the α -naphthylamine method, and

its oxidizing effect on ascorbic acid (or reduced 2:6-dichlorophenol-indophenol) was very poor, but in view of the complex interactions of other constituents with nitrite, this does not disprove that nitrite is responsible for these effects.

As already mentioned, saliva collected from some individuals had little or no effect on ascorbic acid, presumably because nitrite was absent. This ion is believed to be produced by bacterial decomposition of buccal contents (Fearon, 1934) as it is absent from saliva collected from the ducts of the glands, a fact which makes the variation in salivary nitrite more intelligible.

The absence of oxidation by saliva in neutral solution

Since the oxidizing power of nitrites is promoted by an acid medium it seemed possible that no effect was produced during mastication, when the reaction is approximately neutral, but that the large conversion to dehydroascorbic acid occurred after the chewed vegetable had been placed in the metaphosphoric acid. This possibility was tested by taking a solution of ascorbic acid in water and adding 2 ml. of saliva and 0.2 mg. of sodium nitrite to two different portions of 10 ml., a third 10 ml. portion being kept without addition for the measurement of spontaneous loss. Metaphosphoric acid was added after 10 min. and the acidified solutions were titrated within 10 to 15 sec. to determine the loss during the 10 min. standing at neutral pH. Very rapid manipulation and titration were essential as any delay after acidification would have caused oxidation of the ascorbic acid. After 10 min. standing, following the addition of the acid, the solutions were titrated again thus measuring the loss during 10 min. at an acid pH. The rate of spontaneous oxidation was determined by acidifying and titrating portions of the neutral ascorbic acid solution every 5 min. throughout the experiment. A typical result is given in Table 5; such experiments have all shown that neither nitrite nor saliva accelerates the spontaneous loss occurring in a neutral solution of ascorbic acid, but that after addition of metaphosphoric acid, which completely arrested the spontaneous loss, oxidation was rapid.

Table 5. *Effect of nitrite and saliva on ascorbic acid in neutral and metaphosphoric acid solution*

Percentage of ascorbic acid (1 mg. in 10 ml. of water) in presence of oxidation

	No addition	0.2 mg. NaNO_2	2 ml. saliva
(a) during 10 min. at neutral pH			
	29	33	29
(b) further oxidation in 10 min. subsequent to acidification			
	0	50	50

Further proof that no loss occurred during standing at neutral pH was provided by adding nitrite and saliva to portions of a solution of ascorbic acid in water and titrating without addition of acid. Again the loss in the presence of nitrite or saliva did not exceed the spontaneous fall in neutral ascorbic acid solution alone; indeed, in some experiments, saliva and nitrite under these conditions appeared to protect ascorbic acid from spontaneous oxidation.

Hence, at the approximately neutral reaction prevailing during mastication of either cooked or raw vegetables, it is unlikely that any oxidation occurs. There seems no doubt that the oxidation that is observed experimentally occurs after the chewed vegetable is strongly acidified, prior to the titration.

Influence of gastric juice on oxidation by saliva

Although the experiments described in the previous section prove that no oxidation occurs in the mouth during mastication, there remains the possibility that when the chewed mass enters the stomach the acidity of the gastric juice may convert the salivary nitrite to nitrous acid, followed by some oxidation of any ascorbic acid that might be present. It was found that the slow fall in titre occurred when nitrite was added to a solution of ascorbic acid in HCl at pH 2.0, thus proving that gastric juice was sufficiently acid to cause the change.

Samples of gastric juice were obtained by a stomach pump during fasting and also at varying intervals after a meal of thin gruel. To these samples portions of a neutral solution of ascorbic acid were added and the mixtures incubated for an hour, after which they were rapidly titrated. In one subject a fall in titre of 40% was observed after the ascorbic acid had been incubated with resting juice, which presumably contained swallowed saliva. On the other hand, a very different result was obtained with the specimens of gastric juice collected after the meal; in one case no fall whatever occurred in the titre, probably because the saliva had been diluted by the gruel and by the considerable flow of gastric juice which followed the meal. Also since the nitrite is believed to be formed in the mouth and not by the salivary glands, it is probable that the saliva swallowed with the meal contained little or no nitrite as it would not remain in the mouth sufficiently long for nitrite to be produced. Tests have shown that the nitrite content of saliva is much reduced after a meal.

The gastric juice from a second subject was found to reduce the dye rapidly even before ascorbic acid was added. Peters & Martin (1937) have reported that both human and dog's gastric juice contained a substance which reduced 2:6-dichlorophenol indophenol which they assumed to be ascorbic acid itself. These workers found that the reducing sub-

stance was fairly stable in human gastric juice, little loss being observed during 24 hr. standing, while in dog's juice no loss occurred during this period. Similarly in the present work the 'spontaneous' reducing power of the gastric juice was maintained, and added ascorbic acid was stable during a period of 2 hr. observation.

This part of the work has not been pursued further but it would seem that although in some conditions slight oxidation may occur in the stomach, saliva normally produced during a meal does not cause significant oxidation of ascorbic acid, even when acidified by gastric juice. From what follows it will be seen that a fall of titre as high as 40% would in any case represent an irreversible oxidation of less than 10% of the ascorbic acid present. If oxidation does occur in the stomach, ascorbic acid from all dietary sources and not merely from raw vegetables would be liable to be affected.

The relative unimportance of reoxidation of dye during titration

In the experiments in which dehydroascorbic acid was reduced to ascorbic acid by H_2S , the latter had to be thoroughly removed by passing a current of air or nitrogen through the acid solution, a procedure which will also remove nitrite from the extracts. If reoxidation of 2:6-dichlorophenol-indophenol by nitrite is solely, or mainly, responsible for the fall in the ascorbic titre, then no fall would be observed if the nitrite were removed before titration. If, on the other hand, the fall were due to previous oxidation of ascorbic acid to dehydroascorbic acid by the nitrite, then the removal of nitrite subsequent to this oxidation would not affect the result. Aspiration, with air, of an acidified ascorbic acid solution which had stood with saliva, without H_2S treatment (a process which removed nitrite only) was found to have a negligible effect on the titration reading thus confirming the conclusion previously arrived at, that if nitrite is the active substance in saliva, it acts by oxidizing the ascorbic acid in the acid extract rather than by acting on the dye. Further evidence is provided by the observation that after adding nitrite or saliva to an acid solution of ascorbic acid the titration-reading depends on the length of time elapsing between the addition and the titration. If the effect were mainly due to interference with the reduction of the dye the change in titre would be instantaneous.

Absorption from the mouth

Although the action in acidified saliva of an oxidizing substance, almost certainly nitrous acid, explains most of the apparent loss of ascorbic acid during mastication it is probable that it does not account for the whole effect. In the experiments in which the dehydroascorbic acid present in the extracts of chewed vegetables was reduced back to ascorbic acid, it was found that complete restoration of the original amount of ascorbic acid was rarely obtained. In Table 3, for instance, 28 and 38% of the ascorbic acid was unaccounted for either as

surviving ascorbic acid or dehydroascorbic acid. When nitrite or saliva was added to an acid solution of ascorbic acid *in vitro* and allowed to stand, a large fall in ascorbic acid content occurred, but treatment with H_2S restored much of it, usually about 80 %, though the results were variable. These experiments suggest that the nitrous acid in the acid extracts may oxidize a small part of the ascorbic acid irreversibly and this was undoubtedly one of the reasons for the incomplete recoveries shown in Table 4.

The concentration of ascorbic acid in the mouth during these experiments would greatly exceed that of blood and tissues and would favour absorption by the richly vascular buccal mucosa. The absorption of certain drugs from the mouth has been established (e.g. Walton, 1935, 1944) and experiments have been reported which indicate that ascorbic acid may diffuse through stratified epithelium (Kasahara & Kawashima, 1937).

That some diffusion may readily occur is suggested by a number of recovery experiments conducted with citric acid, which is comparable to ascorbic acid in solubility and molecular weight. Crystalline citric acid (10 mg.) was placed in the mouth for 1 min. after which the mouth was thoroughly washed out. In one series of these experiments the average recovery was about 80 % and similar recoveries were obtained when the experiments were repeated with dilute solutions of citric acid.

DISCUSSION

The experiments described clearly demonstrate that a large part of the apparent loss of ascorbic acid during mastication of vegetables is due to its conversion into dehydroascorbic acid by substances in saliva, probably mainly nitrite, and that a small fraction is irreversibly lost.

The oxidation occurs only at a strongly acid pH and does not take place in the mouth during chewing, but while the chewed material is standing in acid, during the course of the analytical procedure. It seems likely, however, that the acidity of gastric juice may promote the oxidation to a limited extent under some conditions and if so, small irreversible losses of ascorbic acid may occur. It seems likely that absorption from the mouth contributes slightly to the observed loss, but this does not result in any real loss of ascorbic acid to the body.

It seems certain that ascorbic oxidase has too

small an influence to be of any practical importance during mastication; indeed in most of the experiments no evidence whatever of its influence could be detected. The fact that the duration of mastication in the experiments was probably at least twice as long as in normal eating reduces still further the likelihood of any appreciable enzymic loss during normal mastication.

SUMMARY

1. It is confirmed that mastication of raw vegetables causes a considerable apparent fall in their ascorbic acid content as judged by subsequent titration in acid solution against indophenol dye. The fact that similar apparent losses occur with cooked as well as with raw vegetables disproves the hitherto accepted theory that ascorbic acid oxidase is responsible.

2. It has been shown that most samples of saliva can slowly oxidize ascorbic acid mainly to dehydroascorbic acid when allowed to stand in acid solution, and, to a less extent, cause a reoxidation of reduced 2:6-dichlorophenol-indophenol. Some irreversible oxidation of ascorbic acid may also occur which is estimated at less than 20 % of the original content. Nitrite is the only constituent of saliva which has been shown to possess these properties though other salivary constituents may modify the effect.

3. Evidence is presented that such oxidation of ascorbic acid in acid medium is responsible for the apparent loss on mastication. The loss, however, does not actually occur till the chewed material is placed in metaphosphoric acid for extraction prior to analysis, and there is no evidence of a real destruction in the mouth under normal conditions, though some, largely reversible, oxidation may occur in the stomach of the ascorbic acid from all dietary sources, and not merely that from raw vegetables.

4. Absorption of ascorbic acid from the mouth probably contributes, but to only a small extent, to the apparent loss.

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