CLXXXIX. THE ENZYMIC DETERMINATION OF ASCORBIC ACID

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CHEMICAL assay of ascorbic acid in plant and animal tissues, using the redox indicator, 2:6-dichlorophenolindophenol is at best only approximate, for the method assumes in general that, apart from ascorbic acid, no other substance with a reduction potential lower than that of 2:6-dichlorophenolindophenol is present in such tissues, whose capacity, therefore, to reduce the indicator is taken to represent their ascorbic acid content. When, however, a tissue is suspected to contain other indophenol-reducing impurities in addition to ascorbic acid, the impurities are sought to be removed by treating the tissue extract with mercuric acetate [Emmerie & Van Eekelen, 1934]; but it is again doubtful whether all such interfering substances can thereby be completely removed. If, on the other hand, ascorbic acid alone in the mixture of indophenol-reducing substances of a tissue extract can be oxidized by any means, the resultant fall in the indophenol titre of the extract can be legitimately equated to its ascorbic acid content.

Such a means of estimation is afforded in the discovery of an enzyme capable of oxidizing ascorbic acid [Szent-Györgyi, 1931; Tauber *et al.* 1935; Srinivasan, 1935; 1936, 1]. The enzyme oxidizes also *d*-gluco-ascorbic acid [Zilva, 1936], which is, however, antiscorbutically inert [Zilva, 1935]. Excepting for this interference, which is highly improbable, from the stereoisomerides of ascorbic acid, enzymic estimation of ascorbic acid appears to be the most rational amongst the chemical methods. At any rate, the enzymic method will undoubtedly be useful in determining, as it is proposed to determine later, the ascorbic acid fraction of the indophenol reduction characterizing tumours, urine, aqueous humour and other biological materials. Also, as it now appears that ascorbic acid oxidase is widely distributed and is simple of isolation [Szent-Györgyi, 1931; Zilva, 1934; Tauber *et al.* 1935; Srinivasan, 1935; 1936, 1, 2; Hopkins & Morgan, 1936; Chakraborty & Guha, 1937], the adoption of the enzyme for estimating ascorbic acid further commends itself.

Tauber & Kleiner [1935] were the first to use ascorbic acid oxidase for estimating its substrate in natural sources and the present investigation is devoted to perfecting this new method. In the method as described by Tauber & Kleiner the extracts of the test tissues were incubated with the enzyme for unduly long periods (30–60 min.), which have been considerably shortened in the present investigation by the employment of an active enzyme preparation: thereby, autoxidation of indophenol-reducing substances in the tissue extracts has also been minimized. Further, in common with other methods, the enzymic estimation of tissue ascorbic acid has now been carried out at room temperature without detriment to the accuracy of the estimation.

EXPERIMENTAL

Methods. An active preparation of ascorbic acid oxidase from drumstick, *Moringa pterygosperma*, was prepared by the method of Srinivasan [1936, 1]. As now obtained, 1 ml. of this enzyme solution was found to oxidize in 5 min. and at room temperature (about 25°) 0.5 mg. of ascorbic acid present in a total volume of 5 ml. of reaction mixture buffered at pH 5.3 (Table I). In all subsequent experiments, therefore, this quantity of enzyme was used under similar conditions.

Table I. *Standardization of ascorbic acid oxidase in terms of 0.5 mg. of ascorbic acid*

Reaction mixture: 0.5 mg. of ascorbic acid and 1 ml. of phosphate buffer of pH 5.3 in a total volume of 5 ml. Reaction carried out at room temperature.

Quantity of enzyme ml.	% ascorbic acid oxidized	
	3 min.	5 min.
0.5	57.0	96.4
1.0	96.4	100.0

An identical experiment conducted as above, but in the presence of trichloroacetate ion, in concentration corresponding to 5% trichloroacetic acid, showed that this acid in its neutralized state does not affect the activity of ascorbic acid oxidase. Hence, in employing the enzyme for the estimation of ascorbic acid in a tissue, the usual trichloroacetic acid extract of the tissue formed the starting point. The neutralized extract, in aliquots corresponding to not more than 0.5 mg. of ascorbic acid (calculated from the indophenol titre), 1 ml. of phosphate buffer of pH 5.3 and suitable quantities of glass-distilled water to make the final volume after the addition of enzyme 5 ml. were treated with 1 ml. of ascorbic acid oxidase from drumstick. Enzyme action was allowed to proceed under vigorous shaking of the reaction mixture at room temperature for 5 min., at the end of which interval the contents of the beaker were acidified and titrated against a standard solution of 2,6-dichlorophenol-indophenol. Control experiments were run under identical conditions, but without the enzyme.

It was then observed, in general, that, while the indophenol-reducing substances of a tissue extract in the control experiment did not undergo any change beyond minor autoxidation effects, the same substances were almost completely oxidized in the solution treated with enzyme.

Specificity of substrate oxidation by the enzyme. It was, however, necessary to show in the first instance that substances other than ascorbic acid in the extract were not oxidized by the enzyme. Such evidence was obtained from experiments conducted under standard conditions with the juice of sour lime, *Citrus bergamia*, in which the fall in iodine equivalent of ascorbic acid after

Table II. *Determination of ascorbic acid in lime juice by ascorbic acid oxidase using both indophenol and iodine for titration*

	mg. ascorbic acid per ml. of juice obtained by	
	Indophenol	Iodine
Original juice	0.71	0.73
Juice after treatment with enzyme under standard conditions	0.29	0.32

treatment with the oxidase was almost identical with the corresponding fall in indophenol value in a duplicate experiment. Hence in subsequent experiments, the diminution in the indophenol titre of an extract after its treatment with the enzyme was taken as a measure of the ascorbic acid content of the extract.

Oxidation of an extract of Indian gooseberry with ascorbic acid oxidase. As a known source of ascorbic acid for checking the content by enzymic oxidation the Indian gooseberry, *Phyllanthus emblica*, was chosen, which, even after treatment with mercuric acetate, was previously found to give a high titre with 2:6-dichlorophenolindophenol [Damodaran & Srinivasan, 1935, 1, 2]. 5 g. of gooseberry pulp were extracted with trichloroacetic acid as usual, the extract finally made up to 100 ml. and the indophenol titre of an aliquot determined. The pH of the extract was then adjusted to 5.3 and a portion of the neutralized extract subjected to mercuric acetate-H₂S treatment. Aliquots of the original and mercury-treated extracts, each corresponding to not more than 0.5 mg. of ascorbic acid (calculated from the indophenol titre) were severally treated with ascorbic acid oxidase under known conditions and, at the end of 5 min. of enzymic oxidation, the reaction mixture was acidified and titrated with the indophenol reagent. Together with the titration of the extract at different stages, the corresponding time of titration, reckoned from the treatment of the material with trichloroacetic acid, was also noted, to find out the extent of autoxidation of the indophenol-reducing substances in the extract.

Experiments with ascorbic acid. As a comparative study, the above series of experiments was repeated under identical conditions with a solution of ascorbic acid (0.46 mg./2 ml.). The values thus obtained in the two sets of experiments with gooseberry and ascorbic acid are recorded together in Table III.

Table III. *Effect of ascorbic acid oxidase on gooseberry extract and ascorbic acid solution before and after mercury treatment*

Treatment	Time of titration reckoned from treatment with trichloroacetic acid min.	mg. of ascorbic acid [*]			Ascorbic equivalent of the enzyme-resistant and Hg-non-precipitable residue in gooseberry extract mg./g.	True ascorbic acid content of gooseberry mg./g.
		Per 2 ml.				
		Solution of ascorbic acid	Gooseberry extract	Per g. of gooseberry extract		
(A) Trichloroacetic acid extract of gooseberry or ascorbic acid titrated both before and after enzyme action	45	0.40	0.44	4.4	0.15	4.25
(B) pH of (A) adjusted to 5.3; supernatant liquid, after centrifuging, titrated just before mercury treatment	95	0.35	0.40	4.0	—	—
(C) 25 ml. of (B) treated with mercuric acetate + H ₂ S, final volume made up to 50 ml. and allowed to stand overnight: then H ₂ S-free aliquot, equivalent to twice that of B (or A), titrated both before and after enzyme treatment	95	0.35	0.38	3.8	0.15	4.25

From the results obtained it was observed that subjecting gooseberry extract and a solution of ascorbic acid of equal strength to similar treatments affected their indophenol-reducing values to almost the same degree. Further, most of the reducing factor in gooseberry was oxidized by ascorbic acid oxidase. Hence, it appears safe to conclude that the original indophenol titre of gooseberry extract nearly represents its ascorbic acid content.

At the end of 5 min. of enzymic action, however, the gooseberry extract, either the original, or, the mercury-treated, in contradistinction to the solution of ascorbic acid, was still capable of reducing, though to a small extent, the indophenol reagent. This indophenol titre persisted even after prolonged and intensive treatment of the extract with ascorbic acid oxidase, which by itself had no effect on indophenol. Thus, the presence in gooseberry of an indophenol-reducing impurity, resistant to enzymic action and not precipitable by mercuric acetate, was clearly indicated. To the extent represented by this small, but persistent indophenol titre, then, should the original apparent ascorbic acid value of the extract be taken to be in excess of the true value.

The extent of substrate oxidation by ascorbic acid oxidase was found to be the same in the mercury-treated extract as in the original, implying that the mercury-precipitable impurities, inclusive of those giving a colour with FeCl_3 , present in gooseberry extract [Damodaran & Srinivasan, 1935, 1, 2], were not necessary for the specific action of the enzyme, nor were they attacked by the enzyme.

There now remains to be explained the observed gradual diminution in the original ascorbic acid value of the gooseberry extract during the course of its various treatments. From the behaviour of the extract compared with that of a solution of ascorbic acid, the lowering in the indophenol-reducing value of the gooseberry extract after mercury treatment must be attributed, not to the removal thereby of indophenol-reducing impurities, as was previously done [Damodaran & Srinivasan, 1935, 1, 2], but to unavoidable losses of ascorbic acid through adsorption on impurities precipitated by mercury [Fischer, 1934; Euler & Malmberg, 1935] and/or by oxidation. The latter cause, induced by the extractants (trichloroacetic acid and water) and atmospheric oxygen, is undoubtedly responsible for the fall in the indophenol titre of ascorbic acid solution, or to a less extent, of the protected gooseberry extract [Damodaran & Ramakrishnan Nair, 1936] during the period preceding the mercuric acetate- H_2S treatment. On the other hand, the small discrepancy between the indophenol-reducing values of gooseberry extract just before and after mercury treatment is an adsorption effect that does not arise in a pure solution of ascorbic acid under similar treatment.

Hence, it becomes increasingly evident that the substance almost wholly responsible for the reduction of indophenol in gooseberry extract is ascorbic acid, the quantity of which in that source has now been correctly assessed with the aid of the oxidase.

Enzymic estimation of ascorbic acid in other sources. The ascorbic acid values, obtained after enzymic action, of materials previously found by the Tillmans titration to be rich sources of the vitamin [Damodaran & Srinivasan, 1935, 1, 2] are given in Table IV, wherein the correction due to extraneous reducing factors

Table IV. *Ascorbic acid content of materials as determined by enzymic action*

Material	Ascorbic acid value before enzyme treatment	Ascorbic acid equivalent of the enzyme-resistant and Hg-non-precipitable impurity	True ascorbic acid value
Drumstick, <i>Moringa pterygosperma</i> , pods	2.80 mg./g.	0.09 mg./g.	2.71 mg./g.
Agati, <i>Sesbania grandiflora</i> , leaf	2.14 mg./g.	0.05 mg./g.	2.09 mg./g.
Orange (Sautgur), <i>Citrus aurantium</i> , juice	0.70 mg./ml.	0.01 mg./ml.	0.69 mg./ml.

has been derived after varying both the time of enzymic action and the quantity of enzyme.

The quantity of indophenol-reducing impurities in the pods of *Moringa pterygosperma* and in the leaf of *Sesbania grandiflora*, as now determined by the resistance of these impurities to enzymic oxidation, was found to be small. A similar result was obtained previously (Damodaran & Srinivasan) from the approximate equality of the ascorbic acid values, as calculated from iodine and indophenol titres of the extracts of these materials.

In contrast to the common variety of orange, which is characterized by considerable quantities of enzyme-resistant indophenol-reducing impurities [cf. Tauber & Kleiner, 1935], the Sautgur orange, a local variety of *Citrus aurantium*, was found to possess very much less of these impurities, suggesting its nearness, in this respect, to the lemon, whose capacity to reduce indophenol is known to be due wholly to ascorbic acid.

DISCUSSION

The ascorbic acid values of the tissues after treating their extracts with the enzyme were repeatedly found to be lower in every case studied than the original values of the extracts. This lowering, which was no doubt small, but persistent, even after prolonged and intensive enzymic treatment of extract purified by precipitation with mercuric acetate, must be entirely ascribed to an impurity in the experimental tissues. That the difference is not due to experimental error is proved by the fact that identical values are obtained before and after enzymic treatment of a solution of pure ascorbic acid.

Similarly, only small quantities of impurities were detected by Tauber & Kleiner [1935] in the sources of ascorbic acid examined by them. It is not unlikely, however, that in materials like urine and tumour extracts, to mention a few instances now believed to abound in indophenol-reducing impurities, the enzymic method will demonstrate the difference between the true ascorbic acid value and that obtained by the usual Tillmans titration. As it is difficult to foresee the presence and the quantity of indophenol-reducing impurities in sources to be tested for ascorbic acid, the enzymic treatment of the test extracts, like the treatment with mercuric acetate- H_2S in some cases, seems to be essential for a correct estimation of ascorbic acid.

SUMMARY

1. Experimental conditions for estimation of tissue ascorbic acid with the aid of its oxidase were determined.
2. On applying this new technique to a few sources of ascorbic acid, the presence therein of small quantities of indophenol-reducing impurities, in addition to ascorbic acid, was detected by the resistance of these impurities to enzymic oxidation. It was also found that mercuric acetate did not precipitate these impurities.
3. The correct amount of ascorbic acid present in a few of its previously known sources was estimated.

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REFERENCES

- Chakraborty & Guha (1937). *Indian J. med. Res.* **24**, 839.
 Damodaran & Ramakrishnan Nair (1936). *Biochem. J.* **30**, 1014.
 ——— & Srinivasan (1935, 1). *Curr. Sci.* **3**, 553.
 ——— (1935, 2). *Proc. Ind. Acad. Sci.* **2 B**, 377.
 Emmerie & Van Eekelen (1934). *Biochem. J.* **28**, 1153.
 Euler & Malmberg (1935). *Arch. Augenheilk.* **109**, 225.
 Fischer (1934). *Klin. Wschr.* **13**, 596.
 Hopkins & Morgan (1936). *Biochem. J.* **30**, 1446.
 Srinivasan (1935). *Curr. Sci.* **4**, 407.
 ——— (1936, 1). *Biochem. J.* **30**, 2077.
 ——— (1936, 2). *Curr. Sci.* **5**, 296.
 Szent-Györgyi (1931). *J. biol. Chem.* **90**, 385.
 Tauber & Kleiner (1935). *J. biol. chem.* **110**, 559.
 ——— & Mishkind (1935). *J. biol. Chem.* **110**, 211.
 Zilva (1934). *Biochem. J.* **28**, 663.
 ——— (1935). *Biochem. J.* **29**, 1612.
 ——— (1936). *Biochem. J.* **30**, 1215.